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UTILITY PATENT APPLICATION TRANSMITTAL

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Philip N. Benfey et al.

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APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

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 - Descriptive title of the Invention
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 - Reference to Microfiche Appendix
 - Background of the Invention
 - Brief Summary of the Invention
 - Brief Description of the Drawings (if filed)
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 - Claim(s)
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The following utility patent application is enclosed for filing:

Applicant(s): Philip N. Benfey *et al.*

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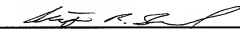
Title of Invention: SHORT-ROOT GENE, PROMOTER, AND USES THEREOF

PATENT APPLICATION FEE VALUE

TYPE	NO. FILED	LESS	EXTRA	EXTRA RATE	FEE
Total Claims	41	-20	21	\$18.00 each	\$ 378.00
Independent	12	-3	9	\$78.00 each	\$ 702.00
Minimum Fee					\$ 690.00
Multiple Dependency Fee If Applicable (\$260.00)					\$ 260.00
Total					\$ 2,030.00
50% Reduction for Independent Inventor, Nonprofit Organization or Small Business Concern (a verified statement as to the applicant's status is attached)					- \$ 0.00
Total Filing Fee					\$ 2,030.00

- ☐ Priority of application no. filed on in is claimed under 35 U.S.C. § 119.
- ☐ The certified copy of the priority application has been filed in application no. filed
- ☐ Amend the specification by inserting before the first line the following sentence: This is a continuation-in-part of application no. filed .

Respectfully submitted,


 Atulya R. Agarwal

 40,887
 (Reg. No.)

 For: Laura A. Coruzzi
 (Reg. No. 30,742)
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Enclosure

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SHORT-ROOT GENE, PROMOTER, AND USES THEREOF

This invention was made with government support under grant number: GM RO1 43778 awarded by the National Institute of Health. The government may have certain rights in the invention.

1. INTRODUCTION

The present invention generally relates to the *SHORT-ROOT (SHR)* gene family and their promoters. The invention more particularly relates to ectopic expression of members of the *SHORT-ROOT* gene family in transgenic plants to artificially modify plant structures. The invention also relates to utilization of the *SHORT-ROOT* promoter for tissue and organ specific expression of heterologous gene products.

2. BACKGROUND OF THE INVENTION

Asymmetric cell divisions, in which a cell divides to give two daughters with different fates, play an important role in the development of all multicellular organisms. In plants, because there is no cell migration, the regulation of asymmetric cell divisions is of heightened importance in determining organ morphology. In contrast to animal embryogenesis, most plant organs are not formed during embryogenesis. Rather, cells that form the apical meristems are set aside at the shoot and root poles. These reservoirs of stem cells are considered to be the source of all post-embryonic organ development in plants. A fundamental question in developmental biology is how meristems function to generate plant organs.

25

2.1. Root Development

Root organization is established during embryogenesis. This organization is propagated during postembryonic development by the root meristem. Following germination, the development of the postembryonic root is a continuous process, wherein a series of initials or stem cells continuously divide to perpetuate the pattern established in the embryonic root (Steeves & Sussex, 1972, Patterns in Plant Development, Englewood Cliffs, NJ: Prentice-Hall, Inc.).

35

2.1.1. Arabidopsis Root Development

Due to the organization of the Arabidopsis root, it is possible to follow the fate of cells from the meristem to maturity and identify the progenitors of each cell type

(Dolan et al., 1993, Development 119:71-84). The Arabidopsis root is a relatively simple and well characterized organ. The radial organization of the mature tissues in the Arabidopsis root has been likened to tree rings with the epidermis, cortex, endodermis and pericycle forming radially symmetric cell layers that surround the vascular cylinder. See also Dolan et al., 1993, Development 119:71-84. These mature tissues are derived from four sets of stem cells or initials: i) the columella root cap initial; ii) the pericycle/vascular initial; iii) the epidermal/lateral root cap initial; and iv) the cortex/endodermal initial (Dolan et al., 1993, Development 119:71-84). It has been shown that these initials undergo asymmetric divisions (Scheres et al., 1995, Development 121:53-62). The cortex/endodermal initial, for example, first divides anticlinally (in a transverse orientation). This asymmetric division produces another initial and a daughter cell. The daughter cell, in turn, expands and then divides periclinally (in the longitudinal orientation). This second asymmetric division produces the progenitors of the endodermis and the cortex cell lineages.

Furthermore, root radial organization in Arabidopsis is produced by three distinct developmental strategies. First, primary roots employ stem cells, wherein initials undergo asymmetric divisions first to regenerate themselves and then to generate the cell lineages of the root. Second, in the embryo, sequential asymmetric divisions subdivide pre-existing tissue to form the cell layers of the embryonic root. Finally, lateral roots are formed by a strategy of cell proliferation that originates in differentiated tissues. Remarkably, within a given species, all three strategies result in roots with a nearly identical radial organization.

2.2. Genes Regulating Root Structure

Mutations that disrupt the asymmetric divisions of the cortex/endodermal initial have been identified and characterized (Benfey et al., 1993, Development 119:57-70; Scheres et al., 1995, Development 121:53-62). *short-root* (*shr*) and *scarecrow* (*scr*) mutants are missing a cell layer between the epidermis and the pericycle. In both types of mutants, the cortex/endodermal initial divides anticlinally, but the subsequent periclinal division that increases the number of cell layers does not take place (Benfey et al., 1993, Development 119:57-70; Scheres et al., 1995, Development 121:53-62). The defect is first apparent in the embryo and it extends throughout the entire embryonic axis, which includes the embryonic root and hypocotyl (Scheres et al., 1995, Development 121:53-62). This is true also for other radial organization mutants characterized to date, suggesting that radial patterning that occurs during embryonic development may influence the post-embryonic

pattern generated by the meristematic initials (Scheres et al., 1995, Development 121:53-62).

In embryos, cortex and endodermis are also formed from the asymmetric division of embryonic ground tissue at the early torpedo stage. This division occurs along the length of the embryonic axis which encompasses the embryonic root and hypocotyl. In both *scarecrow* and *short-root*, the embryonic ground tissue fails to undergo the asymmetric division into cortex and endodermis. Hence, these two mutations identify genes required for the asymmetric division that produces cortex and endodermis from ground tissue in the embryo and from the cortex/endodermal initials in primary and lateral roots.

Characterization of the mutant cell layer in *shr* indicated that two endodermal-specific markers were absent, while the cortex-specific markers were present, indicating that the mutant layer has differentiated attributes only of cortex. Thus, in *short-root* the initial cell divides transversely, then fails to make the longitudinal division and in the resulting cell only the cortex differentiation program is activated. This suggests that the *short-root* mutant phenotype is equivalent to the loss of the endodermal cell layer and distinct from the *scarecrow* phenotype. For *SHORT-ROOT*, the findings suggest that it is involved in specification of endodermis identity and is also directly or indirectly required for the asymmetric division to form cortex and endodermis. (Benfey et al., 1993, Development 119:57-70).

2.3. Role of SHR in Radial Patterning of the Shoot

SHR functions are not confined to the roots. The hypocotyls of SHR mutants are also missing one of the ground tissue derived cell layers (Scheres, B., et al., 1995, Development 121, 53-62; Fukaki et al., 1998, Plant J. 14, 425-430) but the cellular identities of the remaining cell layers are not known. The absence of normal hypocotyl and shoot endodermis correlates with an agravitropic phenotype in both hypocotyl and shoot inflorescence (Fukaki, et al 1998, Plant J. 14, 425-430). Thus, mutations in the *SHR* genes lead to a radial pattern deficiency both in the roots and shoots. However, the pattern defects have no effect on root gravitropism. The data suggests that lack of sedimenting amyloplasts in the *shr* mutant stems is responsible for the shoot agravitropic phenotype (Fukaki, et al, 1998, Plant J. 14, 425-430).

2.4. Geotropism

In plants, the capacity for gravitropism has been correlated with the presence of amyloplast sedimentation. See, e.g., Volkmann and Sievers, 1979, Encyclopedia Plant Physiol., N.S. vol 7, pp. 573-600; Sack, 1991, Intern. Rev. Cytol. 127:193-252; Björkman, 1992, Adv. Space Res. 12:195-201; Poff et al., in The Physiology of Tropisms, Meyerowitz

- & Somerville (eds); Cold Spring Harbor Laboratory Press, Plainview, NY (1994) pp. 639-664; Barlow, 1995, Plant Cell Environ. 18:951-962. Amyloplast sedimentation only occurs in cells in specific locations at distinct developmental stages. That is, when and where sedimentation occurs is precisely regulated (Sack, 1991, Intern. Rev. Cytol. 127:193-252).
- 5 In roots, amyloplast sedimentation only occurs in the central (columella) cells of the rootcap; as these cells mature into peripheral cap cells, the amyloplasts no longer sediment (Sack & Kiss, 1989, Amer. J. Bot. 76:454-464; Sievers & Braun, in The Root Cap: Structure and Function, Wassail et al. (eds.), New York: M. Dekker (1996) pp. 31-49). In stems of many plants, including Arabidopsis, amyloplast sedimentation occurs in the starch
- 10 sheath (endodermis) especially in elongating regions of the stem (von Guttenberg, Die Physiologischen Scheiden, Handbuch der Pflanzenanatomie; K. Linsbauer (ed.), Berlin: Gebruder Borntraeger, vol. 5 (1943) p. 217; Sack, 1987, Can. J. Bot. 65:1514-1519; Sack, 1991, Intern. Rev. Cytol. 127:193-252; Caspar & Pickard, 1989, Planta 177:185-197; Volkmann et al., 1993, J. Pl. Physiol. 142:710-6).
- 15 Gravitropic mutants have been studied for evidence that proves the role of amyloplast sedimentation in gravity sensing. However, many gravitropic mutations affect downstream events such as auxin sensitivity or metabolism (Masson, 1995, BioEssays 17:119-127). Other mutations seem to affect gene products that process information from gravity sensing. For example, the lazy mutants of higher plants and comparable mutants in
- 20 mosses can clearly sense and respond to gravity, but the mutations reverse the normal polarity of the gravitropic response (Gaiser & Lomax, 1993, Plant Physiol. 102:339-344; Jenkins et al., 1986, Plant Cell Environ 9:637-644). Other mutations appear to affect gravitropism of specific organs. For example, *sgf* mutants have defective shoot gravitropism (Fukaki et al., 1996, Plant Physiol. 110:933-943; Fukaki et al., 1996, Plant
- 25 Physiol. 110:945-955; Fukaki et al., 1996, Plant Res. 109:129-137).

Citation or identification of any reference herein shall not be construed as an admission that such reference is available as prior art to the present invention.

3. SUMMARY OF THE INVENTION

- 30 The structure and function of a regulatory gene, *SHORT-ROOT (SHR)*, is described. The *SHR* gene is expressed specifically in root progenitor tissues of embryos, and in certain tissues of roots and stems. More specifically, *SHR* is expressed in tissues internal to the endodermis, namely, the pericycle, phloem, protoxylem and procambium. *SHR* expression controls cell division of certain cell types in roots, and affects the
- 35 organization of root and stem. The present invention relates to the *SHORT-ROOT (SHR)* gene (which encompasses the Arabidopsis *SHR* gene and its orthologs and paralogs), *SHR*-

like genes, *SHR* gene products, (including, but not limited to, transcriptional products such as mRNAs, antisense and ribozyme molecules, and translational products such as the *SHR* protein, polypeptides, peptides and fusion proteins related thereto), antibodies to *SHR* gene products, *SHR* regulatory regions and the use of the foregoing to improve agronomically valuable plants.

The invention is based, in part, on the discovery, identification and cloning of the gene responsible for the *short-root* phenotype. The inventors have determined that the mutant cell layer in roots of *shr* mutants has differentiated characteristics of cortex but not of endodermis. This is consistent with a role for *SHR* in the regulation of asymmetric cell division and in specification of the identity of endodermis. The inventors have determined also that *SHR* expression affects the gravitropism of plant aerial structures such as the shoot.

One aspect of the invention relates to the heterologous expression of *SHR* genes and related nucleotide sequences, and specifically the Arabidopsis *SHR* gene, in stably transformed higher plant species. Modulation of *SHR* expression levels can be used to advantageously modify root and aerial structures of transgenic plants and enhance the agronomic properties of such plants.

Another aspect of the invention relates to the use of promoters of *SHR* genes, and specifically the use of the Arabidopsis *SHR* promoter to control the expression of protein and RNA products in plants. Plant *SHR* promoters have a variety of uses, including, but not limited to, expressing heterologous genes in the embryo, root, root nodule and shoot of transformed plants.

The invention is illustrated by working examples, described *infra*, which demonstrate the expression, cloning and characterization of the Arabidopsis *SHR* gene.

Structural analysis of the deduced amino acid sequence of Arabidopsis *SHR* protein indicates that *SHR* encodes a transcription factor. Promoter marker gene analysis and *in situ* hybridization analysis show highly localized expression of Arabidopsis *SHR* in embryos and roots. Genetic analysis shows *SHR* expression also affects gravitropism of aerial structures (*e.g.*, stems and shoots). This indicates that *SHR* is also expressed in those structures.

The present invention further provides compositions and methods for screening compounds that modulate expression within embryos, roots, and shoots. In particular, it provides compositions comprising nucleotides from the *SHR* promoter, and transcriptionally active fragments thereof, as well as nucleic acids that hybridize under highly stringent conditions to such nucleotides, that control the expression of such *SHR* promoter. Specifically provided are expression vectors comprising the *SHR* promoter, and

transcriptionally active fragments thereof, operably associated to a heterologous reporter gene, and host cells and transgenic plants containing such vectors. The invention also provides methods for using such vectors, cells and plants for screening candidate molecules for agonists and antagonists of the *SHR* promoter.

5 For example, and not by way of limitation, a composition comprising a reporter gene is operatively linked to a *SHR* promoter. The *SHR* driven reporter gene is expressed as a transgene in plants. The transgenic plant, and cells derived from embryos, roots or shoots of such transgenic plant, can be used to screen compounds for candidates useful for modulating a *SHR* promoter. Without being bound by any particular theory, such
10 compounds are likely to interfere with the function of trans-acting factors, such as transcription factors, cis-acting elements, such as promoters and enhancers, as well as any class of post-transcriptional, translational or post-translational compounds involved in modulating a *SHR* promoter.

In another embodiment, the transgenic plant models of the invention can be
15 used for *in vivo* screening to test the mechanism of action of candidate drugs for their effect on root or shoot-related disorders.

In addition to tissue specific promoters, the present invention encompasses vectors using inducible promoters. Inducible promoters have the advantage that they can be switched on and off. Therefore, if a cell is stably transfected with a transgene under the
20 control of an inducible promoter, its expression could be controlled over the life-time of the cell.

The invention further provides methods for screening for novel transcription factors that modulate the *SHR* promoter sequence. Such novel transcription factors identified by this method can be used as targets for treating root or shoot-related disorders.

25 The various embodiments of the claimed invention presented herein are by way of illustration only and are in no manner intended to limit the scope of the invention.

3.1. Definitions

As used herein, the terms listed below will have the meanings indicated.

30 35S = cauliflower mosaic virus promoter for the 35S transcript
cDNA = complementary DNA
cis-regulatory element= A promoter sequence 5' upstream of the TATA box that confers specific regulatory response to a promoter containing
35 such an element. A promoter may contain one or more cis-

		regulatory elements, each responsible for a particular regulatory response
	coding sequence	= sequence that encodes a complete or partial gene product (e.g., a complete protein or a fragment thereof)
5	DNA	= deoxyribonucleic acid
	EST	= expressed sequence tag
	functional portion	= a functional portion of a promoter is any portion of a promoter that is capable of causing transcription of a linked gene sequence, e.g., a truncated promoter
10	gene fusion	= a gene construct comprising a promoter operably linked to a heterologous gene, wherein said promoter controls the transcription of the heterologous gene
	gene product	= the RNA or protein encoded by a gene sequence
15	gene sequence	= sequence that encodes a complete gene product (e.g., a complete protein)
	GUS	= 1,3- β -Glucuronidase
	gDNA	= genomic DNA
20	heterologous gene	= In the context of gene constructs, a heterologous gene means that the gene is linked to a promoter that said gene is not naturally linked to. The heterologous gene may or may not be from the organism contributing said promoter. The heterologous gene may encode messenger RNA (mRNA), antisense RNA or ribozymes
25	homologous promoter	= a native promoter of a gene that selectively hybridizes to the sequence of a <i>SHR</i> gene described herein
	mRNA	= messenger RNA
	operably linked	= A linkage between a promoter and gene sequence such that the transcription of said gene sequence is controlled by said promoter
30	ortholog	= related gene in a different plant
	paralog	= related gene in the same plant
	RNA	= ribonucleic acid
35	RNase	= ribonuclease

SHR (italic) = *SHORT-ROOT* gene or portion thereof, encompasses *SHR* genes and its orthologs and paralogs

SHR = *SHORT-ROOT* protein

shr (lower case, italic) = *short-root* mutant (e.g., *shr1*)

SHR polypeptides and peptides include deleted or truncated forms of the *SHR* protein, and fragments corresponding to the *SHR* motifs described herein.

SHR fusion proteins encompass proteins in which the *SHR* protein or a *SHR* polypeptide or peptide is fused to a heterologous protein, polypeptide or peptide.

SHR gene, nucleotides or coding sequences mean nucleotides, e.g., gDNA or cDNA encoding *SHR* protein, *SHR* polypeptides, peptides or *SHR* fusion proteins.

SHR gene products include transcriptional products such as mRNAs, antisense and ribozyme molecules, as well as translational products of the *SHR* nucleotides described herein, including, but not limited to, the *SHR* protein, polypeptides, peptides and/or *SHR* fusion proteins.

SHR promoter means the regulatory region native to the *SHR* gene in a variety of species, which promotes the organ and tissue specific pattern of *SHR* expression described herein.

4. BRIEF DESCRIPTION OF THE FIGURES

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be understood better by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

Figure 1A-1H. Phenotype of *shr* Mutant Plants. (A) Schematic of Arabidopsis root anatomy (left). Transverse section showing epidermis, cortex, endodermis, and pericycle surrounding the vascular tissue. In the longitudinal section, the epidermal/lateral root cap initials and the cortex/endodermal initials are shown at the base of their respective cell files. Schematic of division pattern of the cortex/endodermal initial (right). The initial expands and then divides anticlinally to reproduce itself and a daughter cell. The daughter then divides periclinally to produce the progenitors of the endodermis and cortex cell lineages. Abbreviations: C, cortex; Da, daughter cell; E, endodermis; In, initial. Similar schematics were in Di Lorenzo et al., 1996 Cell 86, 423-433. (B) 12-day

old wild-type (WT), *shr-1*, *shr-2*, *shr-3*, and *shr-4* seedlings grown vertically on nutrient agar medium. Transverse plastic sections of primary root of (C) wild-type and (D) *shr-2*. CCRC-M2 antibody staining in (E) epidermis and cortex of wild-type and in (B) epidermis and mutant layer of *shr-1* primary roots. AX92::GUS expression in mutant layer of (G) a longitudinal optical section and (H) a transverse section of a *shr-1* primary root.

Abbreviations: ep, epidermis; co, cortex; en, endodermis; m, mutant ground tissue layer in *shr*.

Figure 2A-2C. Molecular Cloning of the SHR Gene. (A) The candidate band of En hybridization cosegregating with the *shr* mutant phenotype is indicated (arrow). A similar size band is found in one of the homozygous wild-type plants (line 52). PCR analysis of the En-SHR junction in this line indicated that the similar molecular weight band does not correspond to the band observed in the homozygous mutants. Numbers above the gel lanes indicate individual plants of the segregating population. Molecular size markers are indicated on the left. (B) Alignment of the deduced amino acid sequence of the "VHIID domains" of five functionally characterized GRAS family genes (*Ls*: Schumacher et al., 1999, Proc. Natl. Acad. Sci. USA 96, 290-295; *GAI*: Peng et al., 1997, Nature 400, 256-261; *RGA*: Silverstone et al., 1998, Plant Cell 10, 155-169; *SCR*: Di Laurenzio et al., 1996 Cell 86, 423-433). Numbers before the sequence indicate the position of the first amino acid of the alignment in the corresponding position in the proteins. Conserved amino acids are shown in bold. *SHR* is not highly similar to any other functionally characterized GRAS gene. (C) Mutation sites in *shr* alleles. Note that *shr-4* has a duplication of nucleotide triplets (TAG; underlined) at the En insertion site, while the En insertion in *shr-3* did not result in an alteration of the host sequence. *shr-1*, which has a 50 nucleotide base pair deletion, has a deletion from the threonine at position 408 and includes the cysteine at position 424 of the native SHR sequence. Thus, the remaining sequence is

GATGAGTTC...ATGGGAAGAGA.

Figure 3A-3G. SHR Expression in the Primary Root. (A) *SHR* RNA accumulation in seedlings. Northern blot analysis was performed with total RNA from 12-day old root tissues hybridized with a *SHR* gene-specific probe. Lanes are as marked. (B and C) In situ RNA hybridization with a *SHR* gene-specific probe. (B) Longitudinal and (C) transverse sections of wild-type primary roots hybridized with a digoxigenin-labeled antisense *SHR* gene-specific probe. Longitudinal optical sections of wild-type roots with (D) confocal laser scanning microscopy of *SHR::GFP* or (E) histochemical staining (0.5 hr)

of *SHR::GUS* reveal *SHR* expression in the stele tissue, not in the ground tissue lineage. Confocal images of *SHR::GFP* expression in (F) *shr-3*, and (G) *shr-1* primary roots. Abbreviations as above except: CEI, cortex/endodermal initial; QC, quiescent center; st, stele.

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Figure 4A-4F. *SHR* Expression during Embryogenesis. Longitudinal view of *SHR::GFP* expression during wild-type embryogenesis. (A) Late globular stage; (B) triangular stage; (C) heart stage; (D) torpedo stage; (E) mature embryonic root; (F) mature embryonic shoot. Abbreviations as above except: cot, cotyledon; hyp, hypophysis; g, ground tissue; gm, ground meristem; my, mid vein of cotyledon; pc, procambium; pd, protoderm; SAM, shoot apical meristem; su, suspensor.

Figure 5A-5C. *SCR* Expression in *shr* Background. (A) *SCR* RNA accumulation in seedling roots. Northern blot analysis was performed with total RNA from 12-day-old wild-type, *shr-1*, and *shr-2* root tissues hybridized with a *SCR* gene-specific probe. The same blot was hybridized with a *GDHI* (Melo-Oliveira et al., 1996, Proc. Natl. Acad. Sci. USA 93, 4718-4723) gene-specific probe as a loading control. (B and C) Expression of *SCR::GFP* in the primary root. GFP expression in (B) wild-type and (C) *shr-2* seedling roots harboring the *SCR::GFP* transgene indicating that the *shr* mutation results in reduced expression from the *SCR* promoter. Abbreviations as above.

Figure 6A-6H. Primary Root Sector in the Unstable *shr-3* Allele. (A, C, and E) Transverse sections and (B, D, F) JIM 13 antibody staining (endodermis and stele marker) of primary roots. (A, B) wildtype; (C, D) *shr-3* (mutant phenotype); (E, F) *shr-3* with a primary root sector. (F) Red arrows indicate the divided ground tissue cells decorated by JIM13. The red star indicates an undivided ground tissue cell decorated by JIM13. (G and H) Expression of the *SCR::GFP* reporter gene in the same *shr-3* seedling with the primary root sector shown in (E and F). Note that the *SCR::GFP* reporter gene is expressed strongly only in one cell file on the right side of the root, which corresponds to the divided cells seen in Figures E and F. Abbreviations as above.

Figure 7A-7E. Effects of ectopic expression of *SHR*. (A) Confocal image of longitudinal optical section of root meristem of 35S::*SHR* seedling. Note supernumerary cell files as compared to wildtype (Figure 5B). (B) Transverse section of 35S::*SHR* seedling root. Compare to wild-type transverse section (Figure 1C). (C) Ectopic *SHR* expression

results in altered cell specification as revealed by JIM 13 antibody staining of cross-section of 35S::SHR root (serial section of that shown in Figure 5B). Compare to wildtype JIM 13 staining (Figure 6B) and *shr* JIM 13 staining (Figure 6D). (D) Expression of *SHR* in 35S::SHR seedling analyzed by in situ hybridization on transverse root section with antisense *SHR* probe (compare to wildtype expression in Figure 3C). (E) Expression of *SCR* in 35S::SHR seedling analyzed by in situ hybridization on transverse root section with antisense *SCR* probe. In contrast to wildtype, in which expression is restricted to the endodermis (Pysh et al., 1999, Plant J. 18, 111-119), expression in 35S::SHR is detected in all tissues except stele and root cap. Abbreviations as above except: sn, supernumerary cell layers.

Figure 8. Nucleotide sequence of the *Arabidopsis thaliana* SHR (SEQ ID NO:1). The sequence is from 1 to 2825 base pairs. The mRNA spans 1199 to 2794.

Figure 9. Deduced amino acid sequence of the *Arabidopsis thaliana* SHR (SEQ ID NO:2). The sequence comprises 531 amino acid residues.

Figure 10. Nucleotide sequence of the *Arabidopsis thaliana* *shr-2* mutant (SEQ ID NO:3). The sequence is from 1 to 2059 base pairs.

Figure 11. Nucleotide sequence of the *Arabidopsis thaliana* SHR promoter (SEQ ID NO:4). The sequence is from 1 to 2502 base pairs.

Figure 12A-12D. Description of the pBI-SHRpro-SHR-GFP and pBIH-SHRpro-TE-SHR-GR constructs. Figure 12A represents a schematic of the constructs. Figures 12B-12D represents the method for preparing the constructs.

Figure 13. Description of the pBIH-SCRpro-SHR construct.

Figure 14. Results of attaching the promoter region from the SCARECROW gene to the coding region of SHORT-ROOT.

Figure 15. Cross sections of hypocotyl and root comparing wildtype and plants with the pBIH-SCRpro-SHR construct.

Figure 16. Staining for the Casparian Strip in wildtype and plants with the pBIH-SCRpro-SHR construct.

5. DETAILED DESCRIPTION OF THE INVENTION

5 The invention relates to the *SHORT-ROOT* (*SHR*) gene; *SHORT-ROOT*-like (*SHL*) genes, *SHR* gene products, including, but not limited to, transcriptional products such as mRNAs, antisense and ribozyme molecules, translational products such as the *SHR* protein, polypeptides, peptides and fusion proteins related thereto; antibodies to *SHR* gene products; *SHR* regulatory regions; and the use of the foregoing to improve agronomically
10 valuable plants.

In summary, the data described herein show the identification of *SHR*, a gene involved in the regulation of a specific asymmetric division, in controlling gravitropic response in aerial structures, and in controlling pattern formation in roots. Sequence analysis shows that the *SHR* protein has many hallmarks of transcription factors. *In situ* and
15 marker line expression studies show that *SHR* is expressed in the tissues internal to the endodermis, namely, the pericycle, phloem and procambium. Together, these findings indicate that the *SHR* gene regulates key events that establish the asymmetric division that generates separate cortex and endodermal cell lineages, and that affect tissue organization of roots.

20 Genetic analysis indicates that *SHR* expression affects gravitropism of plant stems, hypocotyls and shoots. This indicates that *SHR* is expressed also in these aerial structures of plants.

The *SHR* genes and promoters of the present invention have a number of important agricultural uses. The *SHR* promoters of the invention may be used in expression
25 constructs to express desired heterologous gene products only in stele tissues (vasculature and pericycle) in the root of transgenic plants transformed with such constructs. For example, *SHR* promoters may be used to express disease resistance genes such as lysozymes, cecropins, maganins or thionins for anti-bacterial protection, or the pathogenesis-related (PR) proteins such as glucanases and chitinases for anti-fungal
30 protection. *SHR* promoters also may be used to express a variety of pest resistance genes in the aforementioned plant structures and tissues. Examples of useful gene products for controlling nematodes or insects include *Bacillus thuringiensis* endotoxins, protease inhibitors, collagenases, chitinase, glucanases, lectins and glycosidases.

Gene constructs that express or ectopically express *SHR*, and the *SHR*-
35 suppression constructs of the invention, may be used to alter the root and/or stem structure, and the gravitropism of aerial structures of transgenic plants. Since *SHR* regulates root cell

divisions, overexpression of *SHR* can be used to increase division of certain cells in roots and thereby form thicker and stronger roots. Thicker and stronger roots are beneficial in preventing plant lodging. Conversely, suppression of *SHR* expression can be used to decrease cell division in roots and thereby form thinner roots. Thinner roots are more efficient in uptake of soil nutrients. Since *SHR* affects gravitropism of aerial structures, overexpression of *SHR* may be used to develop "straighter" transgenic plants that are less susceptible to lodging.

Further, the *SHR* gene sequence may be used as a molecular marker for a quantitative trait, e.g., a root or gravitropism trait, in molecular breeding of crop plants.

Further, the ability to control the length and internal architecture of plant roots would serve several critical problems of modern agriculture and biotechnology. Federal regulations governing fertilizer use combined with the high energy cost of fertilizer production are forcing the AgBiotech industry to look for alternatives to intensive agriculture practices. One attractive alternative is to engineer plants so that they will be better able to utilize the nutrients found in particular locations. Frequently this means that the plant's root must grow deep into the soil. The problem is that most crop plants today have been bred for relatively shallow roots because that is where all of the nutrients are located when high fertilizer levels are used. If one were able to control the length of a plant's roots and one knew where in the soil particular nutrients are at higher concentration (this is frequently already known), then one could program the plant to grow roots to the proper length for optimal utilization of the natural resources.

Another use for controlled root length is in phytoremediation. Plants are currently being engineered to be used in toxic waste clean-up. For them to work effectively, their roots must penetrate to the sites of the toxic substances. Thus, plants in which roots can be programmed to grow to particular lengths would be of great use in this area of biotechnology. A similar argument could be made for plants used in erosion control.

In the presently described unstable allele of short-root, roots of vastly different sizes growing from the same plant have been obtained. Longer roots have been correlated with restoration of *SHORT-ROOT* activity in some cells in those roots. This indicates that controlling *SHORT-ROOT* gene activity is one means of controlling root length. Thus, armed with the *SHR* sequence disclosed herein, one has the potential of altering root length in specific ways by altering the activity level of the *SHORT-ROOT* gene.

The ability to control of the internal architecture of root and shoot could also have potential for agriculture improvement. The endodermis is a specialized tissue in both root and shoot. In the root, it is the site of formation of the Casparian strip, a water impermeable barrier that forces selective uptake of nutrients and ions. In the shoot, it is the

location of sedimenting amyloplasts which are part of the gravity sensing apparatus. Since SHORT-ROOT expression is necessary for differentiation of endodermal cells in both root and shoot, regulating SHORT-ROOT activity in the root could result in plants better able to assimilate water-soluble nutrients. Regulating SHORT-ROOT activity in the shoot could result in plants that are less liable to fall over ("lodge") as a result of environmental stress.

5.1. Polynucleotides and Nucleic Acids of the Invention

The present invention relates to nucleic acid molecules that encode polypeptides referred to as SHR. In accordance with the invention, any nucleotide sequence which encodes the amino acid sequence of the SHR gene product can be used to generate recombinant molecules which direct the expression of *SHR*. Additionally, the invention also relates to a fusion polynucleotide between a *SHR* coding sequence and a second coding sequence for a heterologous protein.

In order to clone full length homologous cDNA sequences from any species encoding the entire *SHR* cDNA or to clone family members or variant forms such as allelic variants, labeled DNA probes made from fragments corresponding to any part of the cDNA sequences disclosed herein may be used to screen a cDNA library derived from a cell or tissue type believed to express *SHR*, e.g. roots. More specifically, oligonucleotides corresponding to either the 5' or 3' terminus of the coding sequence may be used to obtain longer nucleotide sequences. Briefly, the library may be plated out to yield a maximum of 30,000 pfu for each 150 mm plate. Approximately 40 plates may be screened. The plates are incubated at 37°C until the plaques reach a diameter of 0.25 mm or are just beginning to make contact with one another (3-8 hours). Nylon filters are placed onto the soft top agarose and after 60 seconds, the filters are peeled off and floated on a DNA denaturing solution consisting of 0.4N sodium hydroxide. The filters are then immersed in neutralizing solution consisting of 1M Tris HCl, pH 7.5, before being allowed to air dry. The filters are prehybridized in casein hybridization buffer containing 10% dextran sulfate, 0.5M NaCl, 50mM Tris HCl, pH 7.5, 0.1% sodium pyrophosphate, 1% casein, 1% SDS, and denatured salmon sperm DNA at 0.5 mg/ml for 6 hours at 60°C. The radiolabelled probe is then denatured by heating to 95°C for 2 minutes and then added to the prehybridization solution containing the filters. The filters are hybridized at 60°C for 16 hours. The filters are then washed in 1X wash mix (10X wash mix contains 3M NaCl, 0.6M Tris base, and 0.02M EDTA) twice for 5 minutes each at room temperature, then in 1X wash mix containing 1% SDS at 60°C for 30 minutes, and finally in 0.3X wash mix containing 0.1% SDS at 60°C for 30 minutes. The filters are then air dried and exposed to x-ray film for autoradiography. After developing, the film is aligned with the filters to select a positive plaque. If a single,

isolated positive plaque cannot be obtained, the agar plug containing the plaques will be removed and placed in lambda dilution buffer containing 0.1M NaCl, 0.01M magnesium sulfate, 0.035M Tris HCl, pH 7.5, 0.01% gelatin. The phage may then be replated and rescreened to obtain single, well isolated positive plaques. Positive plaques may be isolated and the cDNA clones sequenced using primers based on the known cDNA sequence. This step may be repeated until a full length cDNA is obtained.

- It may be necessary to screen multiple cDNA libraries from different tissues to obtain a full length cDNA. In the event that it is difficult to identify cDNA clones encoding the complete 5' terminal coding region, an often encountered situation in cDNA cloning, the RACE (Rapid Amplification of cDNA Ends) technique may be used. RACE is a proven PCR-based strategy for amplifying the 5' end of incomplete cDNAs. 5'-RACE-Ready RNA synthesized from human placenta containing a unique anchor sequence is commercially available (Clontech). To obtain the 5' end of the cDNA, PCR is carried out on 5'-RACE-Ready cDNA using the provided anchor primer and the 3' primer. A secondary PCR is then carried out using the anchored primer and a nested 3' primer according to the manufacturer's instructions. Once obtained, the full length cDNA sequence may be translated into amino acid sequence and examined for certain landmarks such as a continuous open reading frame flanked by translation initiation and termination sites, a potential signal sequence and finally overall structural similarity to the *SHR* gene sequences disclosed herein.

Alternatively, a labeled probe may be used to screen a genomic library derived from any organism of interest using appropriate stringent conditions as described, *infra*.

- Isolation of a *SHR* coding sequence or a homologous sequence may be carried out by the polymerase chain reactions (PCR) using two degenerate oligonucleotide primer pools designed on the basis of the *SHR* coding sequences disclosed herein. The template for the reaction may be cDNA obtained by reverse transcription (RT) of mRNA prepared from, for example, plant cell lines or tissues known or suspected to express a *SHR* gene allele.

- The PCR product may be subcloned and sequenced to ensure that the amplified sequences represent the sequences of a *SHR* coding sequence. The PCR fragment may then be used to isolate a full length cDNA clone by a variety of methods. For example, the amplified fragment may be labeled and used to screen a bacteriophage cDNA library. Alternatively, the labeled fragment may be used to isolate genomic clones via the screening of a genomic library.

PCR technology may also be utilized to isolate full length cDNA sequences. For example, RNA may be isolated, following standard procedures, from an appropriate cellular or tissue source. An RT reaction may be performed on the RNA using an oligonucleotide primer specific for the most 5' end of the amplified fragment for the priming of first strand synthesis. The resulting RNA/DNA hybrid may then be "tailed" with guanines using a standard terminal transferase reaction, the hybrid may be digested with RNAase H, and second strand synthesis may then be primed with a poly-C primer. Thus, cDNA sequences upstream of the amplified fragment may easily be isolated.

A cDNA clone of a mutant or allelic variant of the *SHR* gene may be isolated, for example, by using PCR. In this case, the first cDNA strand may be synthesized by hybridizing an oligo-dT oligonucleotide to mRNA isolated from tissue known or suspected to be expressed in a plant putatively carrying the mutant *SHR* allele, and by extending the new strand with reverse transcriptase. The second strand of the cDNA is then synthesized using an oligonucleotide that hybridizes specifically to the 5' end of the normal gene. Using these two primers, the product is then amplified via PCR, cloned into a suitable vector, and subjected to DNA sequence analysis through methods well known to those of skill in the art. By comparing the DNA sequence of the mutant *SHR* allele to that of the normal *SHR* allele, the mutation(s) responsible for the loss or alteration of function of the mutant *SHR* gene product can be ascertained.

Alternatively, a genomic library can be constructed using DNA obtained from a plant suspected of or known to carry a mutant *SHR* allele, or a cDNA library can be constructed using RNA from a tissue known, or suspected, to express a mutant *SHR* allele. An unimpaired *SHR* gene or any suitable fragment thereof may then be labeled and used as a probe to identify the corresponding mutant *SHR* allele in such libraries. Clones containing the mutant *SHR* gene sequences may then be purified and subjected to sequence analysis according to methods well known to those of skill in the art.

Additionally, an expression library can be constructed utilizing cDNA synthesized from, for example, RNA isolated from a tissue known, or suspected, to express a mutant *SHR* allele in a plant suspected of or known to carry such a mutant allele. In this manner, gene products made by the putatively mutant tissue may be expressed and screened using standard antibody screening techniques in conjunction with antibodies raised against the normal *SHR* gene product, as described, below. (For screening techniques, see, for example, Harlow and Lane, eds., 1988, "Antibodies: A Laboratory Manual", Cold Spring Harbor Press, Cold Spring Harbor.)

In cases where a *SHR* mutation results in an expressed gene product with altered function (e.g., as a result of a missense), a polyclonal set of anti-SHR gene product

antibodies are likely to cross-react with the mutant *SHR* gene product. Library clones detected via their reaction with such labeled antibodies can be purified and subjected to sequence analysis according to methods well known to those of skill in the art.

- 5 Functional equivalents of the *SHR* gene product include any plant gene product that regulates plant embryo or root development, or, preferably, that regulates root cell division or root tissue organization, or affects gravitropism of plant aerial structures (e.g., stems and hypocotyls). Functional equivalents of the *SHR* gene product include naturally occurring *SHR* gene products, and mutant *SHR* gene products, whether naturally occurring or engineered.

- 10 As used herein, the terms nucleic acid, polynucleotide and nucleotide are interchangeable and refer to any nucleic acid, whether composed of deoxyribonucleosides or ribonucleosides, and whether composed of phosphodiester linkages or modified linkages such as phosphotriester, phosphoramidate, siloxane, carbonate, carboxymethylester, acetamidate, carbamate, thioether, bridged phosphoramidate, bridged methylene
15 phosphonate, bridged phosphoramidate, bridged phosphoramidate, bridged methylene phosphonate, phosphorothioate, methylphosphonate, phosphorodithioate, bridged phosphorothioate or sultone linkages, and combinations of such linkages.

- The terms nucleic acid, polynucleotide and nucleotide also specifically includes nucleic acids composed of bases other than the five biologically occurring bases
20 (adenine, guanine, thymine, cytosine and uracil). For example, a polynucleotide of the invention might contain at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil,
25 dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-
30 isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

Furthermore, a polynucleotide of the invention may comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

- 5 It is not intended that the present invention be limited by the source of the polynucleotide. The polynucleotide can be from a plant or non-plant, derived from any recombinant source, synthesized *in vitro* or by chemical synthesis. The nucleotide may be DNA or RNA and may exist in a double-stranded, single-stranded or partially double-stranded form.

- Nucleic acids useful in the present invention include, by way of example and not limitation, oligonucleotides such as antisense DNAs and/or RNAs; ribozymes; DNA for gene therapy; DNA and/or RNA chimeras; various structural forms of DNA including single-stranded DNA, double-stranded DNA, supercoiled DNA and/or triple-helix DNA; Z-DNA; and the like. The nucleic acids may be prepared by any conventional means typically used to prepare nucleic acids in large quantity. For example, DNAs and RNAs may be
- 15 chemically synthesized using commercially available reagents and synthesizers by methods that are well-known in the art (*see, e.g.*, Gait, 1985, Oligonucleotide Synthesis: A Practical Approach, IRL Press, Oxford, England). RNAs may be produced in high yield via *in vitro* transcription using plasmids such as SP65 (Promega Corporation, Madison, WI).

- The present invention includes any mRNA transcript encoded by the *SHR*
- 20 genes of the invention, including in particular, mRNA transcripts resulting from alternative splicing or processing of mRNA precursors.

- In some circumstances, as where increased nuclease stability is desired, nucleic acids having modified internucleoside linkages may be preferred. Nucleic acids containing modified internucleoside linkages may also be synthesized using reagents and
- 25 methods that are well known in the art. For example, methods for synthesizing nucleic acids containing phosphonate phosphorothioate, phosphorodithioate, phosphoramidate methoxyethyl phosphoramidate, formacetal, thioformacetal, diisopropylsilyl, acetamidate, carbamate, dimethylene-sulfide ($-\text{CH}_2-\text{S}-\text{CH}_2-$), dimethylene-sulfoxide ($-\text{CH}_2-\text{SO}-\text{CH}_2-$), dimethylene-sulfone ($-\text{CH}_2-\text{SO}_2-\text{CH}_2-$), 2'-O-alkyl, and 2'-deoxy-2'-fluoro phosphorothioate
- 30 internucleoside linkages are well known in the art (*see* Uhlmann *et al.*, 1990, Chem. Rev. 90:543-584; Schneider *et al.*, 1990, Tetrahedron Lett. 31:335 and references cited therein).

- In some embodiments of the present invention, the nucleotide is an α -anomeric nucleotide. An α -anomeric nucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β -units, the strands run parallel to
- 35 each other (Gautier *et al.*, 1987, Nucl. Acids Res. 15:6625-6641). The nucleotide is a 2'-O-

methylribonucleotide (Inoue *et al.*, 1987, Nucl. Acids Res. 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue *et al.*, 1987, FEBS Lett. 215:327-330).

- The nucleic acids may be purified by any suitable means, as are well known in the art. For example, the nucleic acids can be purified by reverse phase or ion exchange HPLC, size exclusion chromatography or gel electrophoresis. Of course, the skilled artisan will recognize that the method of purification will depend in part on the size of the DNA to be purified.

- The nucleic acid itself may act as a therapeutic agent, such as for example an antisense DNA that inhibits mRNA translation, or the nucleic acid may encode a SHR capable of inducing a therapeutic affect upon expression in a plant. These gene products can potentially function as therapeutic molecules in a variety of contexts, for example, as cytokines, chemokines, signaling molecules, membrane proteins, transcription factors, intracellular proteins, cytokine binding proteins, and the like.

- The invention also relates to isolated or purified polynucleotides having at least 12 nucleotides (*i.e.*, a hybridizable portion) of a SHR coding sequence or its complement. In other embodiments, the polynucleotides contain at least 25 (continuous) nucleotides, 50 nucleotides, 100 nucleotides, 150 nucleotides, or 200 nucleotides of a SHR coding sequence, or a full-length SHR coding sequence. Nucleic acids can be single or double stranded. Additionally, the invention relates to polynucleotides that selectively hybridize to a complement of the foregoing coding sequences. In preferred embodiments, the polynucleotides contain at least 12, 25, 50, 100, 150 or 200 nucleotides or the entire length of a SHR coding sequence.

- In a specific embodiment, a polynucleotide which hybridizes to a SHR coding sequence or its complement under conditions of low stringency is provided. By way of example and not limitation, exemplary conditions of low stringency are as follows (Shilo and Weinberg, 1981, Proc. Natl. Acad. Sci. USA 78:6789-6792): Filters containing DNA are pretreated for 6 h at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution with the following modifications: 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml salmon sperm DNA, 10% (wt/vol) dextran sulfate, and 5-20 X 10⁶ cpm ³²P-labeled probe is used. Filters are incubated in hybridization mixture for 18-20 h at 40°C, and then washed for 1.5 h at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.1% SDS. The wash solution is replaced with fresh solution and incubated an additional 1.5 h at 60°C. Filters are blotted dry and exposed for autoradiography. If necessary, filters are washed for a third time at 65-68°C and reexposed to film. Other conditions of low

stringency which may be used are well known in the art (e.g., as employed for cross-species hybridizations).

- In another specific embodiment, a polynucleotide which hybridizes to a SHR coding sequence or its complement under conditions of high stringency is provided. By way of example and not limitation, exemplary conditions of high stringency are as follows:
- 5 Prehybridization of filters containing DNA is carried out for 8 h to overnight at 65°C in buffer composed of 6X SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 µg/ml denatured salmon sperm DNA. Filters are hybridized for 48 h at 65°C in prehybridization mixture containing 100 µg/ml denatured salmon sperm
- 10 DNA and 5-20 X 10⁶ cpm of ³²P-labeled probe. Washing of filters is done at 37°C for 1 h in a solution containing 2X SSC, 0.01% PVP, 0.01% Ficoll, and 0.01% BSA. This is followed by a wash in 0.1X SSC at 50°C for 45 min before autoradiography. Other conditions of high stringency which may be used are well known in the art.

- In another specific embodiment, a polynucleotide which hybridizes to a SHR coding sequence or its complement under conditions of moderate stringency is provided. Exemplary conditions of moderate stringency are as follows: Filters containing DNA are pretreated for 6 h at 55°C in a solution containing 6X SSC, 5X Denhart's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution and 5-20 X 10⁶ cpm ³²P-labeled probe is used. Filters are incubated in
- 20 hybridization mixture for 18-20 h at 55°C, and then washed twice for 30 minutes at 60°C in a solution containing 1X SSC and 0.1% SDS. Filters are blotted dry and exposed for autoradiography. Other conditions of moderate stringency which may be used are well-known in the art.

- Other conditions of high stringency which may be used are well known in the art. In general, for probes between 14 and 70 nucleotides in length the melting temperature (T_m) is calculated using the formula: $T_m(^{\circ}\text{C}) = 81.5 + 16.6(\log[\text{monovalent cations (molar)}]) + 0.41(\% \text{ G+C}) - (500/N)$ where N is the length of the probe. If the hybridization is carried out in a solution containing formamide, the melting temperature is calculated using the equation $T_m(^{\circ}\text{C}) = 81.5 + 16.6(\log[\text{monovalent cations (molar)}]) + 0.41(\% \text{ G+C}) - (0.61\%$
- 30 formamide) - (500/N) where N is the length of the probe. In general, hybridization is carried out at about 20-25 degrees below T_m (for DNA-DNA hybrids) or 10-15 degrees below T_m (for RNA-DNA hybrids).

The invention also encompasses nucleotide sequences that encode a mutant of SHR, peptide fragments of SHR, truncated forms of SHR, and SHR fusion proteins.

- 35 These include, but are not limited to, nucleotide sequences encoding mutant *SHR* proteins

and polypeptides; polypeptides or peptides corresponding to one or more domains of SHR, *e.g.*, the bZIP, VHIID or leucine heptad domains, or portions of these domains; truncated forms of SHR, in which one or more of the domains is deleted; or a truncated, nonfunctional SHR. Nucleotides encoding fusion proteins may include, but are not limited to, full length

5 SHR sequences, truncated forms of SHR, or nucleotides encoding peptide fragments of SHR fused to an unrelated protein or peptide, such as for example, a SHR domain fused to an Ig Fc domain which increases the stability and half life of the resulting fusion protein (*e.g.*, SHR-Ig); or an enzyme such as a fluorescent protein or a luminescent protein which can be used as a marker.

10 The invention encompasses highly related gene homologs, *e.g.*, orthologs and paralogs, of the SHR encoding polynucleotide sequences described above. Highly related gene homologs are polynucleotides encoding proteins that are at least 30% identical, or at least 40% identical, preferably 50% identical, more preferably 60% identical, even more preferably 70% or even 80% identical, and most preferably 90% identical, at the amino acid

15 level to the disclosed SHR proteins.

SHR genes share substantial amino acid sequence similarities at the protein level and nucleotide sequence similarities in their encoding genes. The term "substantially similar" or "substantial similarity" when used herein with respect to two amino acid sequences means that the two sequences have at least 75% identical residues, preferably at

20 least 85% identical residues and most preferably at least 95% identical residues. The same term when used herein with respect to two nucleotide sequences means that the two sequences have at least 70% identical residues, preferably at least 85% identical residues and most preferably at least 95% identical residues. Determining whether two sequences are substantially similar may be carried out using any methodologies known to one skilled

25 in the art, preferably using computer assisted analysis.

To determine the percent identity of two amino acid sequences or of two nucleic acids, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or

30 nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, % identity = # of

35

identical overlapping positions/total # of positions x 100). In one embodiment, the two sequences are the same length.

- The determination of percent identity between two sequences also can be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA* 87:2264-2268, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, *et al.* (1990) *J. Mol. Biol.* 215:403-410. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to a nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to a protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res.* 25:3389-3402. Alternatively, PSI-Blast can be used to perform an iterated search which detects distant relationships between molecules (*Id.*). When utilizing BLAST, Gapped BLAST and PSI-Blast programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used (see <http://www.ncbi.nlm.nih.gov>). Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, (1988) *CABIOS* 4:11-17. Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4 can be used. In an alternate embodiment, alignments can be obtained using the NA_MULTIPLE_ALIGNMENT 1.0 program, using a GapWeight of 5 and a GapLengthWeight of 1.

- The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, typically only exact matches are counted.

Moreover, *SHR* genes show highly localized expression in embryos and, particularly, roots. Such expression patterns may be ascertained by Northern hybridizations and *in situ* hybridizations using antisense probes.

- The invention also encompasses (a) DNA vectors that contain any of the foregoing *SHR* coding sequences and/or their complements (*i.e.*, antisense); (b) DNA expression vectors that contain any of the foregoing *SHR* coding sequences operatively

- associated with a regulatory element that directs the expression of the coding sequences; (c) genetically engineered host cells that contain any of the foregoing *SHR* coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences in the host cell; and (d) genetically engineered host cells that express an endogenous *SHR* gene under the control of an exogenously introduced regulatory element (*i.e.*, gene activation).

5.1.1. Isolation of *SHR* Genes

- The following methods can be used to obtain *SHR* and *SHL* genes and coding sequences from a wide variety of plants, including, but not limited to, *Arabidopsis thaliana*, *Zea mays*, *Nicotiana tabacum*, *Daucus carota*, *Oryza*, *Glycine max*, *Lemna gibba* and *Picea abies*.

- Nucleotide sequences encoding a *SHR* gene, a *SHL* gene or portions thereof may be obtained by PCR amplification of plant genomic DNA or cDNA. Useful cDNA sources include "free" cDNA preparations (*i.e.*, the products of cDNA synthesis) and cloned cDNA in cDNA libraries. Root cDNA preparations or libraries are particularly preferred.

- The amplification may use, as the 5'-primer (*i.e.*, forward primer), a degenerate oligonucleotide that corresponds to a segment of a known *SHR* amino acid sequence, preferably from the amino-terminal region. The 3'-primer (*i.e.*, reverse primer) may be a degenerate oligonucleotide that corresponds to a distal segment of the same known *SHR* amino acid sequence (*i.e.*, carboxyl) to the sequence that corresponds to the 5'-primer). For example, the amino acid sequence of the *Arabidopsis SHR* protein (SEQ ID NO:2) may be used to design useful 5' and 3' primers. Preferably, the primers correspond to segments in the VHIID domain of the *SHR* protein (see FIG. 2). The sequence of the optimal degenerate oligonucleotide probe corresponding to a known amino acid sequence may be determined by standard algorithms known in the art. See for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, Vol 2 (1989).

- Further, for amplification from cDNA sources, the 3'-primer may be an oligonucleotide comprising an 3' oligo(dT) sequence. The amplification also may use as primers nucleotide sequences of *SHR* and *SHL* genes or coding sequences.

- PCR amplification can be carried out, *e.g.*, by use of a Perkin-Elmer Cetus thermal cycler and Taq polymerase (Gene Amp™). One can choose to synthesize several different degenerate primers for use in the PCR reactions. It also is possible to vary the stringency of hybridization conditions used in priming the PCR reactions, to allow for

greater or lesser degrees of nucleotide sequence similarity between the degenerate primers and the corresponding sequences in the cDNA library. One of ordinary skill in the art will know that the appropriate amplification conditions and parameters depend, in part, on the length and base composition of the primers and that such conditions may be determined using standard formulae. Protocols for executing all PCR procedures discussed herein are well known to those skilled in the art, and may be found in references such as Gelfand, 1989, PCR Technology, Principles and Applications for DNA Amplification, H.A. Erlich, ed., Stockton Press, New York; and Current Protocols In Molecular Biology, Vol. 2, Ch. 15, Ausubel et al., eds 1988, New York, Wiley & Sons, Inc.

A PCR amplified sequence may be molecularly cloned and sequenced. The amplified sequence may be utilized as a probe to isolate genomic or cDNA clones of a *SHR* gene, as described below. This, in turn, will permit the determination of a *SHR* gene's complete nucleotide sequence, including its promoter, the analysis of its expression, and the production of its encoded protein, as described *infra*.

A *SHR* or *SHL* gene coding sequence also may be isolated by screening a plant genomic or cDNA library using a *SHR* or *SHL* nucleotide sequence as a hybridization probe. For example, the whole, or a segment, of the Arabidopsis *SHR* nucleotide sequence may be used. Alternatively, a *SHR* or *SHL* gene may be isolated from such libraries using a degenerate oligonucleotide that corresponds to a segment of a *SHR* amino acid sequence as a probe. For example, a degenerate oligonucleotide probe corresponding to a segment of the Arabidopsis *SHR* amino acid sequence may be used.

In preparation of cDNA libraries, total RNA is isolated from plant tissues, preferably roots. Poly(A)+ RNA is isolated from the total RNA, and cDNA prepared from the poly(A)+ RNA, all using standard procedures. See, for example, Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, 2d ed., Vol. 2 (1989). The cDNAs may be synthesized with a restriction enzyme site at their 3'-ends by using an appropriate primer and further have linkers or adaptors attached at their 5'-ends to facilitate the insertion of the cDNAs into suitable cDNA cloning vectors. Alternatively, adaptors or linkers may be attached to the cDNAs after the completion of cDNA synthesis.

In preparation of genomic libraries, plant DNA is isolated and fragments are generated, some of which will encode parts of the whole *SHR* protein. The DNA may be cleaved at specific sites using various restriction enzymes. Alternatively, one may use DNase in the presence of manganese to fragment the DNA, or the DNA can be physically sheared, as for example, by sonication. The DNA fragments can then be separated according to size by standard techniques, including, but not limited to, agarose and

polyacrylamide gel electrophoresis, column chromatography and sucrose gradient centrifugation.

The genomic DNA or cDNA fragments can be inserted into suitable vectors, including, but not limited to, plasmids, cosmids, bacteriophages lambda or T₄, and yeast artificial chromosome (YAC) [See, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989); Glover, D.M(ed.), DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K., Vols. I and II (1985)].

The identity of a cloned or amplified *SHR* gene sequence can be verified by comparing the amino acid sequences of its three open reading frames with the amino acid sequence of a *SHR* gene (e.g., Arabidopsis SHR protein [SEQ ID No:2]). A *SHR* gene or coding sequence encodes a protein or polypeptide whose amino acid sequence is substantially similar to that of a SHR protein or polypeptide. The identity of the cloned or amplified *SHR* gene sequence may be further verified by examining its expression pattern, which should show highly localized expression in the embryo and/or root of the plant from which the *SHR* gene sequence was isolated.

Comparison of the amino acid sequences encoded by a cloned or amplified sequence may reveal that it does not contain the entire *SHR* gene or its promoter. In such a case, the cloned or amplified *SHR* gene sequence may be used as a probe to screen a genomic library for clones having inserts that overlap the cloned or amplified *SHR* gene sequence. A complete *SHR* gene and its promoter may be reconstructed by splicing the overlapping *SHR* gene sequences.

5.2. Products Encoded by The Polynucleotides Disclosed Herein

In accordance with the invention, a *SHR* polynucleotide which encodes full length SHR polypeptides, mutant polypeptides, peptide fragments of SHR, SHR fusion proteins or functional equivalents thereof, may be used to generate recombinant DNA molecules that direct the expression of SHR polypeptides, mutant polypeptides, SHR peptide fragments, SHR fusion proteins or a functional equivalent thereof, in appropriate host cells. Such polynucleotides, as well as other polynucleotides which selectively hybridize to at least a part of such *SHR* polynucleotides or their complements, may also be used to produce SHR polypeptides or they may be used in nucleic acid hybridization assays, such as Southern and Northern blot analyses, etc. The polypeptide products encoded by such polynucleotides may be naturally occurring or altered by molecular manipulation of the coding sequence.

Moreover, SHR proteins, polypeptides and peptide fragments, mutated, truncated or deleted forms of SHR and/or SHR fusion proteins can be prepared for a variety of uses, including, but not limited to, the generation of antibodies, as reagents in assays, the identification of other cellular gene products involved in regulation of root development; etc.

Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially the same or functionally equivalent SHR amino acid sequences may be used in the practice of the invention for the cloning and expression of SHR proteins. Such DNA sequences include those which are capable of hybridizing to the *SHR* coding sequences disclosed herein or their complementary sequences under low, moderate or high stringency conditions as described in Section 5.1.

SHR translational products include, but are not limited to, those proteins and polypeptides encoded by the *SHR* gene sequences described in Section 5.1, above. The invention encompasses proteins that are functionally equivalent to the *SHR* gene products described in Section 5.1. Such a *SHR* gene product may contain one or more deletions, additions or substitutions of SHR amino acid residues within the amino acid sequence encoded by any one of the *SHR* gene sequences described, above, in Section 5.1, but which result in a silent change, thus producing a functionally equivalent *SHR* gene product.

Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine; positively charged (basic) amino acids include arginine, lysine and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

"Functionally equivalent", as utilized herein, refers to a protein capable of exhibiting a substantially similar *in vivo* activity as the endogenous *SHR* gene products encoded by the *SHR* gene sequences described in Section 5.1, above. Alternatively, "functionally equivalent" may refer to peptides capable of regulating gene expression in a manner substantially similar to the way in which the corresponding portion of the endogenous *SHR* gene product would.

The nucleotide sequences of the invention may be engineered in order to alter a *SHR* coding sequence for a variety of ends, including but not limited to, alterations which modify processing and expression of the gene product. For example, mutations may be introduced using techniques which are well known in the art, *e.g.*, site-directed mutagenesis, to insert new restriction sites, to alter glycosylation patterns, phosphorylation,

etc. Alterations may also affect one or more biologic activities of SHR. For example, cysteine residues can be deleted or substituted with another amino acid to eliminate disulfide bridges.

- Based on the domain organization of the SHR protein, a large number of
- 5 SHR mutant polypeptides can be constructed by rearranging the nucleotide sequences that encode the SHR domains.

- In another embodiment of the invention, a *SHR* coding sequence, a modified SHR coding sequence or a truncated *SHR* coding sequence corresponding to a specific domain may be ligated to a heterologous sequence to produce a fusion protein. For
- 10 example, for screening of peptide libraries for molecules that bind SHR, it may be useful to encode a chimeric SHR protein expressing a heterologous epitope that is recognized by a commercially available antibody. A fusion protein may also be engineered to contain a cleavage site located between a SHR sequence and the heterologous protein sequence, so that the SHR may be cleaved and separated from the heterologous moiety. A heterologous
- 15 moiety includes, but is not limited to, immunoglobulin constant domain which prolongs *in vivo* half life of the fusion protein, a cell surface molecule which anchors the fusion protein to the cell membrane, and a detectable label such as a fluorescent protein or an enzyme.

- In a specific embodiment of the invention, the nucleotide sequence of *SHR* could be synthesized in whole or in part, using chemical methods well known in the art.
- 20 See, for example, Caruthers *et al.*, 1980, *Nuc. Acids Res. Symp. Ser.* 7:215-233; Crea and Horn, 1980, *Nuc. Acids Res.* 9(10):2331; Matteucci and Caruthers, 1980, *Tetrahedron Letter* 21:719; and Chow and Kempe, 1981, *Nuc. Acids Res.* 9(12):2807-2817. Alternatively, the polypeptide itself could be produced using chemical methods to synthesize a SHR amino acid sequence in whole or in part. For example, peptides can be synthesized by solid phase
- 25 techniques, cleaved from the resin, and purified by preparative high performance liquid chromatography. (*e.g.*, see Creighton, 1983, *Proteins Structures And Molecular Principles*, W.H. Freeman and Co., N.Y. pp. 50-60). The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (*e.g.*, the Edman degradation procedure; see Creighton, 1983, *Proteins, Structures and Molecular Principles*, W.H. Freeman and
- 30 Co., N.Y., pp. 34-49).

- In a specific embodiment of the invention, a polypeptide containing at least 10 (continuous) amino acids of the SHR protein is provided. In other embodiments, the polypeptide may contain at least 20 or 50 amino acids. In specific embodiments, such polypeptides do not contain more than 100, 150 or 200 amino acids. Derivatives or analogs
- 35 of the polypeptides include, but are not limited to, molecules containing regions that are substantially homologous to the SHR protein or fragments thereof (*e.g.*, in various

embodiments, at least 60% or 70% or 80% or 90% or 95% identity over an amino acid sequence of identical size or when compared to an aligned sequence in which the alignment is done by a computer homology program known in the art) or product encoded by a polynucleotide that is capable of hybridizing to a naturally-occurring coding sequence, under highly stringent, moderately stringent, or low stringent conditions. Percent homology may be determined, for example, by comparing sequence information using the BLAST or GAP programs described *supra*.

The present invention also encompasses SHR polypeptides that are coded for by alternatively spliced SHR mRNA transcripts.

The derivatives and analogs of SHR protein can be produced by various methods known in the art. The manipulations which result in their production can occur at the nucleic acid or protein level. For example, a cloned coding sequence can be modified by any of numerous strategies known in the art (Maniatis, T., 1990, Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*. In the production of a polynucleotide encoding a derivative or analog, care should be taken to ensure that the modified coding sequence remains within the same translational reading frame as the antigen, uninterrupted by translational stop signals, in the coding region where the functional domain is encoded.

Additionally, the coding sequence can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Any technique for mutagenesis known in the art can be used, including but not limited to, chemical mutagenesis, *in vitro* site-directed mutagenesis (Hutchinson, C., et al., 1978, J. Biol. Chem 253:6551), use of TAB® linkers (Pharmacia), and the like.

The invention also encompasses mutant SHR proteins and polypeptides that are not functionally equivalent to the gene products described in Section 5.1. Such a mutant SHR protein or polypeptide may contain one or more deletions, additions or substitutions of SHR amino acid residues within the amino acid sequence encoded by any one the SHR gene sequences described above in Section 5.1., and which result in loss of one or more functions of the SHR protein (e.g., recognition of a specific nucleic sequence, binding of a transcription factor, etc.), thus producing a SHR gene product not functionally equivalent to the wild-type SHR protein.

While random mutations can be made to *SHR* DNA (using random mutagenesis techniques well known to those skilled in the art) and the resulting mutant SHRs tested for activity, site-directed mutations of the *SHR* gene and/or coding sequence can be engineered (using site-directed mutagenesis techniques well known to those skilled in the art) to generate mutant SHRs with increased function, (e.g., resulting in improved root formation), or decreased function (e.g., resulting in suboptimal root function).

Manipulations may also be made at the protein level. Included within the scope of the invention are protein fragments or other derivatives or analogs which are differentially modified during or after translation, e.g., by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a heterologous polypeptide or another antigen. Any of numerous chemical modifications may be carried out by known techniques, including but not limited to, specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, NaBH₄; acetylation, formylation, oxidation, reduction; metabolic synthesis in the presence of tunicamycin; etc.

In addition, analogs and derivatives can be chemically synthesized. Non-classical amino acids (i.e., amino acids not encoded by the genetic code) or chemical amino acid analogs can be introduced as a substitution or addition into the sequence. Non-classical amino acids include, but are not limited to, the D-isomers of the common amino acids, α -amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, γ -Abu, ϵ -Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β -alanine, fluoro-amino acids, designer amino acids such as β -methyl amino acids, α -methyl amino acids, $N\alpha$ -methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

In a specific embodiment, the derivative is a chimeric or fusion protein containing SHR or a fragment thereof joined at its amino- or carboxy-terminus to a heterologous protein via a peptide bond. Alternatively, the proteins are connected by a flexible polylinker such as Gly-Cys-Gly or Gly-Gly-Gly-Gly-Ser repeated 1 to 3 times (Bird *et al.*, 1988, Science 242:423-426; Chaudhary *et al.*, 1990, Proc. Nat'l. Acad. Sci. U.S.A. 87:1066-1070). In one embodiment, such a chimeric protein is produced by recombinant expression of a nucleic acid encoding the protein (a *SHR* coding sequence joined in-frame to a coding sequence for another antigen or a heterologous protein). Such a chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and

expressing the chimeric product by methods commonly known in the art. Alternatively, such a chimeric product may be made by protein synthetic techniques, *e.g.*, by use of a peptide synthesizer. Chimeric genes comprising portions of the *SHR* coding sequence fused to any other coding sequences may be constructed.

- 5 In another specific embodiment, the derivative is a molecule comprising a region of homology with *SHR*. By way of example, in various embodiments, a protein region can be considered "homologous" to a second protein region when the amino acid sequence of the first region is at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 90%, or 95% identical, when compared to any sequence in the second region of an equal number of amino
10 acids as the number contained in the first region or when compared to an aligned sequence of the second region that has been aligned by a computer homology program known in the art *e.g.*, the BLAST program described above.

5.3. Production of *SHR* Polypeptides

- 15 While the *SHR* polypeptides and peptides can be chemically synthesized (*e.g.*, see Creighton, 1983, *Proteins: Structures and Molecular Principles*, W.H. Freeman & Co., N.Y.) large polypeptides derived from *SHR* and the full length *SHR* may advantageously be produced by recombinant DNA technology using techniques well known to those skilled in the art for expressing nucleic acid sequences.

- 20 Methods which are well known to those skilled in the art can be used to construct expression vectors containing the *SHR* coding sequence and appropriate transcriptional/translational control signals. These methods include *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* recombination/genetic recombination. (See, for example, the techniques described in Sambrook *et al.*, 1989, *Molecular Cloning A Laboratory Manual*, Cold Spring Harbor Laboratory, N.Y. and Ausubel *et al.*, 1989, *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley Interscience, N.Y.). RNA capable of encoding *SHR* polypeptide may also be chemically synthesized
25 (Gait, ed., 1984, *Oligonucleotide Synthesis*, IRL Press, Oxford).

- A variety of host-expression vector systems may be utilized to express the
30 *SHR* gene products of the invention. Such host-expression systems represent vehicles by which the *SHR* gene products of interest may be produced and subsequently recovered and/or purified from the culture or plant (using purification methods well known to those skilled in the art), but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, exhibit the *SHR* protein of the invention *in situ*.
35

These include, but are not limited to, microorganisms such as bacteria (*e.g.*, *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing SHR protein coding sequences; yeast (*e.g.*, *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing the SHR protein coding sequences; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing the SHR protein coding sequences; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing *SHR* protein coding sequences; or mammalian cell systems (*e.g.*, COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5K promoter; the cytomegalovirus promoter/enhancer; etc.).

Depending on the host/vector system utilized, any of a number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used in the expression vector. For example, when cloning in bacterial systems, inducible promoters such as pL of bacteriophage λ , plac, ptrp, ptac (ptrp-lac hybrid promoter; cytomegalovirus promoter) and the like may be used; when cloning in insect cell systems, promoters such as the baculovirus polyhedron promoter may be used; when cloning in plant cell systems, promoters derived from the genome of plant cells (*e.g.*, heat shock promoters; the promoter for the small subunit of RUBISCO; the promoter for the chlorophyll α/β binding protein) or from plant viruses (*e.g.*, the 35S RNA promoter of CaMV; the coat protein promoter of TMV) may be used; when cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used; when generating cell lines that contain multiple copies of the *SHR* coding sequence, SV40-, BPV- and EBV-based vectors may be used with an appropriate selectable marker.

30 3.3.1. Expression Systems

In bacterial systems a number of expression vectors may be advantageously selected depending upon the use intended for the expressed SHR product. For example, when large quantities of SHR protein are to be produced for the generation of antibodies, screening peptide libraries or formulating pharmaceutical compositions, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include but are not limited to the *E. coli* expression vector

pUR278 (Ruther *et al.*, 1983, EMBO J. 2:1791), in which the *SHR* coding sequence may be ligated into the vector in frame with the *lacZ* coding region so that a hybrid protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety.

In one such embodiment of a bacterial system, full length cDNA sequences are appended with in-frame Bam HI sites at the amino terminus and Eco RI sites at the carboxyl terminus using standard PCR methodologies (Innis *et al.*, 1990, *supra*) and ligated into the pGEX-2TK vector (Pharmacia, Uppsala, Sweden). The resulting cDNA construct contains a kinase recognition site at the amino terminus for radioactive labeling and glutathione S-transferase sequences at the carboxyl terminus for affinity purification (Nilsson, *et al.*, 1985, EMBO J. 4: 1075; Zabeau and Stanley, 1982, EMBO J. 1: 1217).

The recombinant constructs of the present invention may include a selectable marker for propagation of the construct. For example, a construct to be propagated in bacteria preferably contains an antibiotic resistance gene, such as one that confers resistance to kanamycin, tetracycline, streptomycin or chloramphenicol. Suitable vectors for propagating the construct include plasmids, cosmids, bacteriophages or viruses, to name but a few.

In addition, the recombinant constructs may include plant-expressible, selectable or screenable marker genes for isolating, identifying or tracking plant cells transformed by these constructs. Selectable markers include, but are not limited to, genes that confer antibiotic resistance, (*e.g.*, resistance to kanamycin or hygromycin) or herbicide resistance (*e.g.*, resistance to sulfonylurea, phosphinothricin or glyphosate). Screenable markers include, but are not limited to, genes encoding β -glucuronidase (Jefferson, 1987, Plant Mol. Biol. Rep. 5:387-405), luciferase (Ow *et al.*, 1986, Science 234:856-859) and B protein that regulates anthocyanin pigment production (Goff *et al.*, 1990, EMBO J 9:2517-2522).

In embodiments of the present invention which utilize the *Agrobacterium tumefaciens* system for transforming plants, the recombinant constructs may additionally comprise at least the right T-DNA border sequences flanking the DNA sequences to be transformed into the plant cell. Alternatively, the recombinant constructs may comprise the

right and left T-DNA border sequences flanking the DNA sequence. The proper design and construction of such T-DNA based transformation vectors are well known to those skilled in the art.

- In yeast, a number of vectors containing constitutive or inducible promoters may be used (Current Protocols in Molecular Biology, Vol. 2, 1988, Ed. Ausubel et al., Greene Publish. Assoc. & Wiley Interscience, Ch. 13; Grant et al., 1987, Expression and Secretion Vectors for Yeast, in Methods in Enzymology, Eds. Wu & Grossman, 1987, Acad. Press, N.Y., Vol. 153, pp. 516-544; Glover, 1986, DNA Cloning, Vol. II, IRL Press, Wash., D.C., Ch. 3; and Bitter, 1987, Heterologous Gene Expression in Yeast, Methods in Enzymology, Eds. Berger & Kimmel, Acad. Press, N.Y., Vol. 152, pp. 673-684; and The Molecular Biology of the Yeast *Saccharomyces*, 1982, Eds. Strathern et al., Cold Spring Harbor Press, Vols. I and II).

- In cases where plant expression vectors are used, the expression of the *SHR* coding sequence may be driven by any of a number of promoters. For example, viral promoters such as the 35S RNA and 19S RNA promoters of CaMV (Brisson et al., 1984, *Nature* 310:511-514), or the coat protein promoter of TMV (Takamatsu et al., 1987, *EMBO J.* 6:307-311) may be used; alternatively, plant promoters such as the small subunit of RUBISCO (Coruzzi et al., 1984, *EMBO J.* 3:1671-1680; Broglie et al., 1984, *Science* 224:838-843); or heat shock promoters, e.g., soybean hsp17.5-E or hsp17.3-B (Gurley et al., 1986, *Mol. Cell. Biol.* 6:559-565) may be used. These constructs can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, microinjection, electroporation, etc. (Weissbach & Weissbach, 1988, *Methods for Plant Molecular Biology*, Academic Press, NY, Section VIII, pp. 421-463; and Grierson & Corey, 1988, *Plant Molecular Biology*, 2d Ed., Blackie, London, Ch. 7-9).

- An alternative expression system which could be used to express *SHR* is an insect system. In one such system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The *SHR* coding sequence may be cloned into non-essential regions (for example the polyhedron gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedron promoter). Successful insertion of the *SHR* coding sequence will result in inactivation of the polyhedron gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedron gene). These recombinant viruses are then used to infect *Spodoptera frugiperda* cells in which the inserted gene is expressed (e.g., see Smith et al., 1983, *J. Virol.* 46:584; Smith, U.S. Patent No. 4,215,051).

In mammalian host cells, a number of viral based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the *SHR* coding sequence may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing SHR in infected hosts (*e.g.*, See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 81:3655-3659). Alternatively, a vector derived from vaccinia virus can be used, which would typically make use of the vaccinia 7.5K promoter (*See, e.g.*, Mackett *et al.*, 1982, Proc. Natl. Acad. Sci. USA 79:7415-7419; Mackett *et al.*, 1984, J. Virol. 49:857-864; Panicali *et al.*, 1982, Proc. Natl. Acad. Sci. USA 79:4927-4931). Reglatable expression vectors such as the tetracycline repressible vectors may also be used to express the coding sequences in a controlled fashion.

Specific initiation signals may also be required for efficient translation of inserted *SHR* coding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where the entire *SHR* gene, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of the *SHR* coding sequence is inserted, exogenous translational control signals, including the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the *SHR* coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (*see* Bittner *et al.*, 1987, Methods in Enzymol. 153:516-544).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the SHR protein may

be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with the *SHR* coding sequence controlled by appropriate expression control elements (e.g., promoter and/or enhancer sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of foreign DNA, genetically engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the SHR protein. Such engineered cell lines are particularly useful in screening for molecules or drugs that affect SHR function.

A number of selection systems may be used, including but not limited to, the herpes simplex virus thymidine kinase (Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 22:817) genes can be employed in tk⁻, hgp⁺ or apt⁻ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for *dhfr*, which confers resistance to methotrexate (Wigler, et al., 1980, Proc. Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); *gpt*, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); *neo*, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and *hygro*, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30:147) genes. Additional selectable genes include *trpB*, which allows cells to utilize indole in place of tryptophan; *hisD*, which allows cells to utilize histinol in place of histidine (Hartman & Mulligan, 1988, Proc. Natl. Acad. Sci. USA 85:8047); *ODC* (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue L., 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.) and glutamine synthetase (Bebington et al., 1992, Biotech 10:169).

The expression characteristics of an endogenous *SHR* gene within a cell line, plant or microorganism may be modified by inserting a heterologous DNA regulatory element into the genome of a stable cell line, plant or cloned microorganism such that the inserted regulatory element is operatively linked with the endogenous *SHR* gene. For example, an endogenous *SHR* gene which is normally "transcriptionally silent", i.e., an *SHR* gene which is normally not expressed, or is expressed only at very low levels in a cell line, plant or microorganism, may be activated by inserting a regulatory element which is capable

of promoting the expression of a normally expressed gene product in that cell line, plant or microorganism. Alternatively, a transcriptionally silent, endogenous *SHR* gene may be activated by insertion of a promiscuous regulatory element that works across cell types.

- 5 A heterologous regulatory element may be inserted into a stable cell line, plant or cloned microorganism, such that it is operatively linked with an endogenous *SHR* gene, using techniques which are well known to those of skill in the art, such as targeted homologous recombination (*e.g.*, in Chappel, U.S. Patent No. 5,272,071; PCT publication No. WO 91/06667, published May 16, 1991).

10 **5.3.2. Protein Purification**

Once a recombinant protein is expressed, it can be identified by assays based on the physical or functional properties of the product, including radioactive labeling of the product followed by analysis by gel electrophoresis, radioimmunoassay, ELISA, bioassays, etc.

- 15 Once the encoded protein is identified, it may be isolated and purified by standard methods including chromatography (*e.g.*, high performance liquid chromatography, ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. The actual conditions used will depend, in part, on factors such as net charge, hydrophobicity, hydrophilicity, etc., and will be apparent to those having skill in the art. The functional properties may be evaluated using any suitable assay, *e.g.* an assay for the ability to alter root formation. For the practice of the present invention, it is preferred that the polypeptide is at least 80% purified from other proteins. It is more preferred that they are at least 90% purified. For *in vivo* administration, it is preferred that it is greater than 95% purified, and
25 more preferably greater than 99%.

5.4. Identification of Cells That Express SHR

- The host cells which contain the coding sequence and which express a *SHR* gene product, fragments thereof, or a *SHR* fusion protein may be identified by at least four
30 general approaches; (a) DNA-DNA or DNA-RNA hybridization; (b) the presence or absence of "marker" gene functions; (c) assessing the level of transcription as measured by the expression of *SHR* mRNA transcripts in the host cell; and (d) detection of the gene product as measured by its biological activity or by use of analytical techniques such mass-spectroscopy, amino acid sequencing or immunodetection. Prior to the identification of
35 gene expression, the host cells may be first mutagenized in an effort to increase the level of expression of *SHR*, especially in cell lines that produce low amounts of *SHR*.

In the first approach, the presence of the *SHR* coding sequence inserted in the expression vector can be detected by DNA-DNA or DNA-RNA hybridization using probes comprising nucleotide sequences that are homologous to the *SHR* coding sequence or portions or derivatives thereof.

5 In the second approach, the recombinant expression vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, resistance to methotrexate, transformation phenotype, occlusion body formation in baculovirus, etc.). For example, if the *SHR* coding sequence is inserted within a marker gene sequence of the
10 vector, recombinants containing the *SHR* coding sequence can be identified by the absence of the marker gene function. Alternatively, a marker gene can be placed in tandem with the *SHR* coding sequence under the control of the same or different promoter used to control the expression of the *SHR* coding sequence. Expression of the marker in response to induction or selection indicates expression of the *SHR* coding sequence.

15 In the third approach, transcriptional activity for the *SHR* coding region can be assessed by hybridization assays. For example, RNA can be isolated and analyzed by Northern blot using a probe homologous to the *SHR* coding sequence or particular portions thereof. Alternatively, total nucleic acids of the host cell may be extracted and assayed for hybridization to such probes. Additionally, RT-PCR may be used to detect low levels of
20 gene expression.

In the fourth approach, the expression of the SHR protein product can be assessed immunologically, for example by Western blots, immunoassays such as radioimmuno-precipitation, enzyme-linked immunoassays and the like. This can be achieved by using an anti-SHR antibody. Expression of the SHR protein product can also
25 be assessed using analytical techniques such as amino acid sequencing, which can be accomplished by means of, for example, Edman degradation or tandem mass spectroscopy, or by analysis of the masses of peptides generated by partial hydrolysis of the protein product using mass spectroscopy. In the identification of SHR protein by mass spectroscopy, it will often be desirable to separate the SHR protein from other protein
30 constituents of the cell by means of two-dimensional gel electrophoresis, partially hydrolyze the isolated protein using an amino acid specific protease (e.g., Lys-C, trypsin), and then determine the mass of the resulting peptide fragments using mass spectroscopy. Determination of peptide mass can then be used to identify the protein as SHR, or a variant thereof, using a database of the predicted masses of protein proteolysis products and
35 analysis software such as Protein Prospector, which is publicly available on the internet at <http://prospector.ucsf.edu>.

5.4.1. Antibodies to SHR Proteins and Polypeptides

Antibodies that specifically recognize one or more epitopes of SHR, or epitopes of conserved variants of SHR, or peptide fragments of the SHR are also encompassed by the invention. Such antibodies include, but are not limited to, polyclonal antibodies, monoclonal antibodies (mAbs), human, humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab')₂ fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies and epitope-binding fragments of any of the above.

For the production of antibodies, various host animals may be immunized by injection with the SHR protein, a SHR peptide (*e.g.*, one corresponding to a functional domain of the protein), a truncated SHR polypeptide (SHR in which one or more domains has been deleted), functional equivalents of the SHR protein or mutants of the SHR protein. Such SHR proteins, polypeptides, peptides or fusion proteins can be prepared and obtained as described, *supra*. Host animals may include, but are not limited to, rabbits, mice and rats, to name but a few. Various adjuvants may be used to increase the immunological response, depending on the host species, including, but not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*. Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of the immunized animals.

Monoclonal antibodies to SHR may be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein, (Nature, 1975, 256:495-497), the human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today, 4:72; Cote et al., 1983, Proc. Natl. Acad. Sci., 80:2026-2030) and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including, but not limited to, IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the monoclonal antibodies of this invention may be cultivated *in vitro* or *in vivo*.

Additionally, recombinant antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, are within the scope of the invention. A chimeric antibody is a molecule in which different portions are derived from different species, such as those having a variable region derived from a murine mAb and a human

immunoglobulin constant region. (See, e.g., Cabilly *et al.*, U.S. Patent No. 4,816,567; and Boss *et al.*, U.S. Patent No. 4,816,397, which are incorporated herein by reference in their entirety.) Humanized antibodies are antibody molecules from non-human species having one or more complementarily determining regions (CDRs) from the non-human species and a framework region from a human immunoglobulin molecule. (See, e.g., Queen, U.S. Patent No. 5,585,089, which is incorporated herein by reference in its entirety.) Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in PCT Publication No. WO 87/02671; European Patent Application 184,187; European Patent Application 171,496; European Patent Application 173,494; PCT Publication No. WO 86/01533; U.S. Patent No. 4,816,567; European Patent Application 125,023; Better *et al.* (1988) *Science* 240:1041-1043; Liu *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:3439-3443; Liu *et al.* (1987) *J. Immunol.* 139:3521-3526; Sun *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:214-218; Nishimura *et al.* (1987) *Canc. Res.* 47:999-1005; Wood *et al.* (1985) *Nature* 314:446-449; and Shaw *et al.* (1988) *J. Natl. Cancer Inst.* 80:1553-1559; Morrison (1985) *Science* 229:1202-1207; Oi *et al.* (1986) *Bio/Techniques* 4:214; U.S. Patent 5,225,539; Jones *et al.* (1986) *Nature* 321:552-525; Verhoeven *et al.* (1988) *Science* 239:1534; and Beidler *et al.* (1988) *J. Immunol.* 141:4053-4060.

Completely human antibodies are particularly desirable for therapeutic treatment of human patients. Such antibodies can be produced, for example, using transgenic mice which are incapable of expressing endogenous immunoglobulin heavy and light chains genes, but which can express human heavy and light chain genes. The transgenic mice are immunized in the normal fashion with a selected antigen, e.g., all or a portion of a polypeptide of the invention. Monoclonal antibodies directed against the antigen can be obtained using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA and IgE antibodies. For an overview of this technology for producing human antibodies, see Lonberg and Huszar (1995, *Int. Rev. Immunol.* 13:65-93). For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, see, e.g., U.S. Patent 5,625,126; U.S. Patent 5,633,425; U.S. Patent 5,569,825; U.S. Patent 5,661,016; and U.S. Patent 5,545,806. In addition, companies such as Abgenix, Inc. (Fremont, CA), can be engaged to provide

human antibodies directed against a selected antigen using technology similar to that described above.

- 5 Completely human antibodies which recognize a selected epitope can be generated using a technique referred to as "guided selection." In this approach a selected non-human monoclonal antibody, *e.g.*, a mouse antibody, is used to guide the selection of a completely human antibody recognizing the same epitope. (Jespers *et al.* (1994) *Bio/technology* 12:899-903).

- 10 Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778; Bird, 1988, Science 242:423-426; Huston *et al.*, 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; and Ward *et al.*, 1989, Nature 334:544-546) can be adapted to produce single chain antibodies against SHR proteins or polypeptides. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

- 15 Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, such fragments include, but are not limited to: the F(ab')₂ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')₂ fragments. Alternatively, Fab expression libraries may be constructed (Huse *et al.*, 1989, Science, 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with
20 the desired specificity.

- Antibodies to a SHR protein and/or polypeptide can, in turn, be utilized to generate anti-idiotypic antibodies that "mimic" SHR, using techniques well known to those skilled in the art. (See, *e.g.*, Greenspan & Bona, 1993, FASEB J 7(5):437-444; and Nissinoff, 1991, J. Immunol. 147(8):2429-2438).

25

5.4.2. *SHR* Gene or Gene Products as Markers for Quantitative Trait Loci

- Any of the nucleotide sequences and/or polypeptides described above, can be used as markers for quantitative trait loci in breeding programs for crop plants. To this end,
30 the nucleic acid molecules, including, but not limited to, full length *SHR* coding sequences, and/or partial sequences, can be used in hybridization and/or DNA amplification assays to identify the endogenous *SHR* genes, *shr* mutant alleles and/or *SHR* expression products in cultivars as compared to wild-type plants. They can be used also as markers for linkage analysis of quantitative trait loci. It is possible also that the *SHR* gene may encode a product
35 responsible for a qualitative trait that is desirable in a crop breeding program. Alternatively,

the *SHR* protein, peptides and/or antibodies can be used as reagents in immunoassays to detect expression of the *SHR* gene in cultivars and wild-type plants.

5.4.3. *SHR*-Like Genes

Short-root-like (*SHL*) genes are genes which show a high degree of similarity to the *SHR* gene. Sequence analysis of *SHL* genes showed that a variable amino-terminal (N-terminal) and a highly conserved carboxyl-termini (C-termini) region exist throughout these putative gene products. The highly conserved region does not show significant similarity to members of any recognized gene family, indicating that these sequences likely define a novel gene family. Based on the high degree of similarity of the gene products to *SHR*, the genes corresponding to these sequences were designated SHORT-ROOT-LIKE (*SHL*). Recently, the importance of this gene family has been confirmed. Two components of the gibberellin signal transduction pathway, the gene products of the GIBBERELLIN-ACID INSENSITIVE (*GAI*) and the REPRESSOR OF *GAI* (*RGA*) loci, have been shown to be members of this family (Peng et al., 1997, *Genes & Dev.* 11, 3194-3205; Silverstone et al., 1998, *Plant Cell* 10, 155-169). Thus, this family of gene products has been designated as the GRAS gene family, an acronym based on the designations of the known genes: *GAI*, *RGA*, and *SCR*. An alignment of various GRAS gene products shows that the gene products have at least five recognizable motifs that are highly conserved: the VHIID and SAW motifs, the hydrophobic residues of the leucine heptads, the P-F-Y-R-E residues of the PFYRE motif, and the two short sequences that define the end of the VHIID motif and the beginning of the PFYRE motif. Intriguingly, the majority of the *SHL* genes are expressed predominantly in the root. In addition to root, many of the *SHL* genes are expressed in siliques and shoots.

The *SHL* genes and gene products may be isolated and expressed with methods similar to those discussed for *SHR* genes, *supra*. Furthermore, antibodies to *SHL* proteins and polypeptides may be produced as was discussed, *supra*. Finally, *SHL* genes and gene products may be used as markers for quantitative trait loci as was discussed, *supra*.

5.5. *SHR* Promoters

According to the present invention, *SHR* promoters and functional portions thereof described herein refer to regions of the *SHR* gene which are capable of promoting tissue-specific expression in embryos, roots and shoots of an operably linked coding sequence in plants. The *SHR* promoter described herein refers to the regulatory elements of *SHR* genes, *i.e.*, regulatory regions of genes which are capable of selectively hybridizing to

the nucleic acids described in Section 5.1, or regulatory sequences contained therein, or which are homologous by sequence analysis (containing a span of 10 or more nucleotides in which at least 50 percent of the nucleotides are identical to the sequences presented herein). Homologous nucleotide sequences refer to nucleotide sequences including, but not limited to, *SHR* promoters in diverse plant species (e.g., promoters of orthologs of Arabidopsis *SHR*) as well as genetically engineered derivatives of the promoters described herein.

Methods which could be used for the synthesis, isolation, molecular cloning, characterization and manipulation of *SHR* promoter sequences are well known to those skilled in the art. See, e.g., the techniques described in Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd. ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989).

According to the present invention, *SHR* promoter sequences or portions thereof described herein may be obtained from appropriate plant or mammalian sources from cell lines or recombinant DNA constructs containing *SHR* promoter sequences, and/or by chemical synthetic methods. *SHR* promoter sequences can be obtained from genomic clones containing sequences 5' upstream of *SHR* coding sequences. Such 5' upstream clones may be obtained by screening genomic libraries using *SHR* protein coding sequences, particularly those encoding *SHR* N-terminal sequences, from *SHR* gene clones obtained as described above. Standard methods that may be used in such screening include, for example, the method set forth in Benton & Davis, 1977, Science 196:180 for bacteriophage libraries; and Grunstein & Hogness, 1975, Proc. Nat. Acad. Sci. U.S.A. 72:3961-3965 for plasmid libraries.

The full extent and location of *SHR* promoters within such 5' upstream clones may be determined by the functional assay described below. In the event a 5' upstream clone does not contain the entire *SHR* promoter as determined by the functional assay, the insert DNA of the clone may be used to isolate genomic clones containing sequences further 5' upstream of the *SHR* coding sequences. Such further upstream sequences can be spliced on to existing 5' upstream sequences and the reconstructed 5' upstream region tested for functionality as a *SHR* promoter (i.e., promoting tissue-specific expression in embryos and/or roots of an operably linked gene in plants). This process may be repeated until the complete *SHR* promoter is obtained.

The location of the *SHR* promoter within genomic sequences 5' upstream of the *SHR* gene isolated as described above may be determined using any method known in the art. For example, the 3' end of the promoter may be identified by locating the transcription initiation site, which may be determined by methods such as RNase protection (e.g., Liang et al., 1989, J. Biol. Chem. 264:14486-14498), primer extension (e.g.,

Weissenborn & Larson, 1992, J. Biol. Chem. 267:6122-6131) and/or reverse transcriptase/PCR. The location of the 3' end of the promoter may be confirmed by sequencing and computer analysis, examining for the canonical AGGA or TATA boxes of promoters that are typically 50-60 base pairs (bp) and 25-35 bp, respectively, 5' upstream of the transcription initiation site. The 5' end promoter may be defined by deleting sequences from the 5' end of the promoter containing fragment, constructing a transcriptional or translational fusion of the resected fragment and a reporter gene and examining the expression characteristics of the chimeric gene in transgenic plants. Reporter genes that may be used to such ends include, but are not limited to, GUS, CAT, luciferase, β -galactosidase and C1 and R gene controlling anthocyanin production.

According to the present invention, a *SHR* promoter is one that confers to an operably linked gene in a transgenic plant tissue-specific expression in roots, root nodules, stems and/or embryos. A *SHR* promoter comprises the region between about -5,000 bp and +1 bp upstream of the transcription initiation site of a *SHR* gene. In a particular embodiment, the Arabidopsis *SHR* promoter comprises the region between positions about -2.5 kb and +1 in the 5' upstream region of the Arabidopsis *SHR* gene.

5.5.1. Cis-regulatory Elements of *SHR* Promoters

According to the present invention, the cis-regulatory elements within a *SHR* promoter may be identified using any method known in the art. For example, the location of cis-regulatory elements within an inducible promoter may be identified using methods such as DNase or chemical footprinting (e.g., Meier et al., 1991, Plant Cell 3:309-315) or gel retardation (e.g., Weissenborn & Larson, 1992, J. Biol. Chem. 267:6122-6131; Beato, 1989, Cell 56:335-344; Johnson et al., 1989, Ann. Rev. Biochem. 58:799-839). Additionally, resectioning experiments also may be employed to define the location of the cis-regulatory elements. For example, an inducible promoter-containing fragment may be resected from either the 5' or 3' end using restriction enzyme or exonuclease digests.

To determine the location of cis-regulatory elements within the sequence containing the inducible promoter, the 5' or 3' resected fragments, internal fragments to the inducible promoter containing sequence or inducible promoter fragments containing sequences identified by footprinting or gel retardation experiments may be fused to the 5' end of a truncated plant promoter, and the activity of the chimeric promoter in transgenic plant examined. Useful truncated promoters to these ends comprise sequences starting at or about the transcription initiation site and extending to no more than 150 bp 5' upstream. These truncated promoters generally are inactive or are only minimally active. Examples of such truncated plant promoters may include, among others, a "minimal" CaMV 35S

promoter whose 5' end terminates at position -46 bp with respect to the transcription initiation site (Skriver et al., Proc. Natl. Acad. Sci. USA 88:7266-7270); the truncated "-90 35S" promoter in the X-GUS-90 vector (Benfey & Chua, 1989, Science 244:174-181); a truncated "-101 nos" promoter derived from the nopaline synthase promoter (Aryan et al., 1991, Mol. Gen. Genet. 225:65-71); and the truncated maize Adh-1 promoter in pADcat 2 (Ellis et al., 1987, EMBO J. 6:11-16).

According to the present invention, a cis-regulatory element of a *SHR* promoter is a sequence that confers to a truncated promoter tissue-specific expression in embryos, stems, root nodules and/or roots.

10

5.5.2. *SHR* Promoter-driven Expression Vectors

The properties of the nucleic acid sequences are varied as are the genetic structures of various potential host plant cells. In the preferred embodiments of the present invention, described herein, a number of features which an artisan may recognize as not being absolutely essential, but clearly advantageous are used. These include methods of isolation, synthesis or construction of gene constructs, the manipulation of the gene constructs to be introduced into plant cells, certain features of the gene constructs, and certain features of the vectors associated with the gene constructs.

Further, the gene constructs of the present invention may be encoded on DNA or RNA molecules. According to the present invention, it is preferred that the desired, stable genotypic change of the target plant be effected through genomic integration of exogenously introduced nucleic acid construct(s), particularly recombinant DNA constructs. Nonetheless, according to the present invention, such genotypic changes also can be effected by the introduction of episomes (DNA or RNA) that can replicate autonomously and that are somatically and germinally stable. Where the introduced nucleic acid constructs comprise RNA, plant transformation or gene expression from such constructs may proceed through a DNA intermediate produced by reverse transcription.

The present invention provides for use of recombinant DNA constructs which contain tissue-specific and developmental-specific promoter fragments and functional portions thereof. As used herein, a functional portion of a *SHR* promoter is capable of functioning as a tissue-specific promoter in the embryo, stem, root nodule and/or root of a plant. The functionality of such sequences can be readily established by any method known in the art. Such methods include, for example, constructing expression vectors with such sequences and determining whether they confer tissue-specific expression in the embryo, stem, root nodule and/or root to an operably linked gene.

5 The *SHR* promoters of the invention may be used to direct the expression of any desired protein, or to direct the expression of a RNA product, including, but not limited to, an "antisense" RNA or ribozyme. Such recombinant constructs generally comprise a native *SHR* promoter or a recombinant *SHR* promoter derived therefrom, ligated to the nucleic acid sequence encoding a desired heterologous gene product.

10 A recombinant *SHR* promoter is used herein to refer to a promoter that comprises a functional portion of a native *SHR* promoter or a promoter that contains native promoter sequences that is modified by a regulatory element from a *SHR* promoter. Alternatively, a recombinant inducible promoter derived from the *SHR* promoter may be a chimeric promoter, comprising a full-length or truncated plant promoter modified by the attachment of one or more *SHR* cis-regulatory elements.

15 The manner of chimeric promoter constructions may be any well known in the art. For examples of approaches that can be used in such constructions, see Section 5.1.2., above and Fluhr et al., 1986, Science 232:1106-1112; Ellis et al., 1987, EMBO J. 6:11-16; Strittmatter & Chua, 1987, Proc. Natl. Acad. Sci. USA 84:8986-8990; Poulsen & Chua, 1988, Mol. Gen. Genet. 214:16-23; Comai et al., 1991, Plant Mol. Biol. 15:373-381; Aryan et al., 1991, Mol. Gen. Genet. 225:65-71.

20 According to the present invention, where a *SHR* promoter or a recombinant *SHR* promoter is used to express a desired protein, the DNA construct is designed so that the protein coding sequence is ligated in phase with the translational initiation codon downstream of the promoter. Where the promoter fragment is missing 5' leader sequences, a DNA fragment encoding both the protein and its 5' RNA leader sequence is ligated immediately downstream of the transcription initiation site. Alternatively, an unrelated 5' RNA leader sequence may be used to bridge the promoter and the protein coding sequence.

25 In such instances, the design should be such that the protein coding sequence is ligated in phase with the initiation codon present in the leader sequence, or ligated such that no initiation codon is interposed between the transcription initiation site and the first methionine codon of the protein.

30 Further, it may be desirable to include additional DNA sequences in the protein expression constructs. Examples of additional DNA sequences include, but are not limited to, those encoding: a 3' untranslated region; a transcription termination and polyadenylation signal; an intron; a signal peptide (which facilitates the secretion of the protein); or a transit peptide (which targets the protein to a particular cellular compartment such as the nucleus, chloroplast, mitochondria or vacuole).

35

5.6. Production of Transgenic Plants And Plant Cells

According to the present invention, a desirable plant or plant cell may be obtained by transforming a plant cell with the nucleic acid constructs described herein. In some instances, it may be desirable to engineer a plant or plant cell with several different gene constructs. Such engineering may be accomplished by transforming a plant or plant cell with all of the desired gene constructs simultaneously. Alternatively, the engineering may be carried out sequentially. That is, transforming with one gene construct, obtaining the desired transformant after selection and screening, transforming the transformant with a second gene construct, and so on.

In an embodiment of the present invention, *Agrobacterium* is employed to introduce the gene constructs into plants. Such transformations preferably use binary *Agrobacterium* T-DNA vectors (Bevan, 1984, Nuc. Acid Res. 12:8711-8721) and the co-cultivation procedure (Horsch et al., 1985, Science 227:1229-1231). Generally, the *Agrobacterium* transformation system is used to engineer dicotyledonous plants (Bevan et al., 1982, Ann. Rev. Genet. 16:357-384; Rogers et al., 1986, Methods Enzymol. 118:627-641). The *Agrobacterium* transformation system also may be used to transform, as well as transfer, DNA to monocotyledonous plants and plant cells (see Hernalsteen et al., 1984, EMBO J 3:3039-3041; Hooykass-Van Slogteren et al., 1984, Nature 311:763-764; Grimsley et al., 1987, Nature 325:1677-179; Boulton et al., 1989, Plant Mol. Biol. 12:31-40; Gould et al., 1991, Plant Physiol. 95:426-434).

In other embodiments, various alternative methods for introducing recombinant nucleic acid constructs into plants and plant cells also may be utilized. These other methods are particularly useful where the target is a monocotyledonous plant or plant cell. Alternative gene transfer and transformation methods include, but are not limited to, protoplast transformation through calcium-, polyethylene glycol (PEG), electroporation-mediated uptake of naked DNA (see Paszkowski et al., 1984, EMBO J 3:2717-2722, Potrykus et al., 1985, Mol. Gen. Genet. 199:169-177; Fromm et al., 1985, Proc. Natl. Acad. Sci. USA 82:5824-5828; Shimamoto, 1989, Nature 338:274-276) and electroporation of plant tissues (D'Halluin et al., 1992, Plant Cell 4:1495-1505). Additional methods for plant cell transformation include microinjection, silicon carbide mediated DNA uptake (Kaepler et al., 1990, Plant Cell Reporter 9:415-418) and microparticle bombardment (see Klein et al., 1988, Proc. Natl. Acad. Sci. USA 85:4305-4309; Gordon-Kamm et al., 1990, Plant Cell 2:603-618).

According to the present invention, a wide variety of plants may be engineered for the desired physiological and agronomic characteristics described herein using the nucleic acid constructs of the instant invention and the various transformation

methods mentioned above. In preferred embodiments, target plants for engineering include, but are not limited to, crop plants such as maize, wheat, rice, soybean, tomato, tobacco, carrots, peanut, potato, sugar beets, sunflower, yam, Arabidopsis, rape seed and petunia; and trees such as spruce.

- 5 According to the present invention, desired plants and plant cells may be obtained by engineering the gene constructs described herein into a variety of plant cell types, including, but not limited to, protoplasts, tissue culture cells, tissue and organ explants, pollen, embryos as well as whole plants. In an embodiment of the present invention, the engineered plant material is selected or screened for transformants (*i.e.*, those
- 10 that have incorporated or integrated the introduced gene construct(s)) following the approaches and methods described below. An isolated transformant may then be regenerated into a plant. Alternatively, the engineered plant material may be regenerated into a plant, or plantlet, before subjecting the derived plant, or plantlet, to selection or screening for the marker gene traits. Procedures for regenerating plants from plant cells,
- 15 tissues or organs, either before or after selecting or screening for marker gene(s), are well known to those skilled in the art.

- A transformed plant cell, callus, tissue or plant may be identified and isolated by selecting or screening the engineered plant material for traits encoded by the marker genes present on the transforming DNA. For instance, selection may be performed by
- 20 growing the engineered plant material on media containing inhibitory amounts of the antibiotic or herbicide to which the transforming marker gene construct confers resistance. Further, transformed plants and plant cells also may be identified by screening for the activities of any visible marker genes (*e.g.*, the β -glucuronidase, luciferase, B or C1 genes) that may be present on the recombinant nucleic acid constructs of the present invention.
- 25 Such selection and screening methodologies are well known to those skilled in the art.

- Physical and biochemical methods also may be used to identify a plant or plant cell transformant containing the gene constructs of the present invention. These methods include, but are not limited to: 1) Southern analysis or PCR amplification for detecting and determining the structure of the recombinant DNA insert; 2) Northern blot, S-
- 30 l RNase protection, primer-extension or reverse transcriptase-PCR amplification for detecting and examining RNA transcripts of the gene constructs; 3) enzymatic assays for detecting enzyme or ribozyme activity, where such gene products are encoded by the gene construct; 4) protein gel electrophoresis, western blot techniques, immunoprecipitation, or enzyme-linked immunoassays, where the gene construct products are proteins; 5)
- 35 biochemical measurements of compounds produced as a consequence of the expression of the introduced gene constructs. Additional techniques, such as *in situ* hybridization, enzyme

staining, and immunostaining also may be used to detect the presence or expression of the recombinant construct in specific plant organs and tissues. The methods for doing all of these assays are well known to those skilled in the art.

5.6.1. Transgenic Plants That Ectopically Express *SHR*

In accordance with the present invention, a plant that expresses a recombinant *SHR* gene may be engineered by transforming a plant cell with a gene construct comprising a plant promoter operably associated with a sequence encoding a *SHR* protein or a fragment thereof. (Operably associated is used herein to mean that transcription controlled by the "associated" promoter would produce a functional messenger RNA, whose translation would produce the enzyme.) The plant promoter may be constitutive or inducible. Useful constitutive promoters include, but are not limited to, the CaMV 35S promoter, the T-DNA mannopine synthetase promoter and their various derivatives. Useful inducible promoters include, but are not limited to, the promoters of ribulose biphosphate carboxylase (RUBISCO) genes, chlorophyll a/b binding protein (CAB) genes, heat shock genes, the defense responsive gene (*e.g.*, phenylalanine ammonia lyase genes), wound induced genes (*e.g.*, hydroxyproline rich cell wall protein genes), chemically-inducible genes (*e.g.*, nitrate reductase genes, gluconase genes, chitinase genes, PR-1 genes *etc.*), dark-inducible genes (*e.g.*, asparagine synthetase gene (Coruzzi and Tsai, U.S. Patent 5,256,558, October 26, 1993, Gene Encoding Plant Asparagine Synthetase)) and developmentally regulated genes (*e.g.*, Shoot Meristemless gene), to name just a few.

In yet another embodiment of the present invention, it may be advantageous to transform a plant with a gene construct operably linking a modified or artificial promoter to a sequence encoding a *SHR* protein or a fragment thereof. Typically, such promoters, constructed by recombining structural elements of different promoters, have unique expression patterns and/or levels not found in natural promoters. *See, e.g.*, Salina et al., 1992, Plant Cell 4:1485-1493, for examples of artificial promoters constructed from combining cis-regulatory elements with a promoter core.

In a preferred embodiment of the present invention, the associated promoter is a strong and root, root nodule, stem and/or embryo-specific plant promoter such that the *SHR* protein is overexpressed in the transgenic plant. Examples of root- and root nodules-specific promoters include, but are not limited to, the promoters of *SCR* genes, *SHR* genes, leghemoglobin genes, nodulin genes and root-specific glutamine synthetase genes (*See e.g.*, Tingey et al., 1987, EMBO J. 6:1-9; Edwards et al., 1990, Proc. Nat. Acad. Sci. USA 87:3459-3463).

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In yet another preferred embodiment of the present invention, the overexpression of *SHR* protein in roots may be engineered by increasing the copy number of the *SHR* gene. One approach to producing such transgenic plants is to transform with nucleic acid constructs that contain multiple copies of the complete *SHR* gene (*i.e.*, with its own native *SHR* promoter). Another approach is to repeatedly transform successive generations of a plant line with one or more copies of the complete *SHR* gene. Yet another approach is to place a complete *SHR* gene in a nucleic acid construct containing an amplification-selectable marker (ASM) gene such as the glutamine synthetase or dihydrofolate reductase gene. Cells transformed with such constructs are subjected to culturing regimes that select cell lines with increased copies of complete *SHR* genes. *See, e.g.*, Donn et al., 1984, J. Mol. Appl. Genet. 2:549-562, for a selection protocol used to isolate a plant cell line containing amplified copies of the GS gene. Because the desired gene is closely linked to the ASM, cell lines that amplify the ASM gene are likely also to have amplified the *SHR* gene. Cell lines with amplified copies of the *SHR* gene can then be regenerated into transgenic plants.

5.6.2. Transgenic Plants That Suppress Endogenous *SHR* Expression

In accordance with the present invention, a desired plant may be engineered by suppressing *SHR* activity. In one embodiment, the suppression may be engineered by transforming a plant with a gene construct encoding an antisense RNA or ribozyme complementary to a segment, or the whole, of the *SHR* RNA transcript, including the mature target mRNA. In another embodiment, *SHR* gene suppression may be engineered by transforming a plant cell with a gene construct encoding a ribozyme that cleaves the *SHR* mRNA transcript. Alternatively, the plant can be engineered, *e.g.*, via targeted homologous recombination, to inactive or "knock-out" expression of the plant's endogenous *SHR*.

For all of the aforementioned suppression constructs, it is preferred that such gene constructs express specifically in the root, root nodule, stem and/or embryo tissues. Alternatively, it may be preferred to have the suppression constructs expressed constitutively. Thus, constitutive promoters, such as the nopaline and the CaMV 35S promoter, also may be used to express the suppression constructs. A most preferred promoter for these suppression constructs is a *SCR* or *SHR* promoter.

In accordance with the present invention, desired plants with suppressed target gene expression may be engineered also by transforming a plant cell with a co-suppression construct. A co-suppression construct comprises a functional promoter operatively associated with a complete or partial *SHR* gene sequence. It is preferred that the

operatively associated promoter be a strong, constitutive promoter, such as the CaMV 35S promoter. Alternatively, the co-suppression construct promoter can be one that expresses with the same tissue and developmental specificity as the *SHR* gene.

- According to the present invention, it is preferred that the co-suppression
- 5 construct encodes an incomplete *SHR* mRNA, although a construct encoding a fully functional *SHR* mRNA or enzyme also may be useful in effecting co-suppression.

- In accordance with the present invention, desired plants with suppressed target gene expression also may be engineered by transforming a plant cell with a construct that can effect site-directed mutagenesis of the *SHR* gene. (See, e.g., Offringa et al., 1990,
- 10 EMBO J. 9:3077-84; and Kanevskii et al., 1990, Dokl. Akad. Nauk. SSSR 312:1505-1507 for discussions of nucleic constructs for effecting site-directed mutagenesis of target genes in plants.) It is preferred that such constructs effect suppression of the *SHR* gene by replacing the endogenous *SHR* gene sequence through homologous recombination with either none, or inactive *SHR* protein coding sequences. In other embodiments, antisense,
- 15 gene "knock-out," ribozyme, RNA interference (RNA-i) and/or triple helix methods can be used to affect *SHR* expression.

- Antisense RNA and DNA molecules act to directly block the translation of mRNA by hybridizing to targeted mRNA and preventing protein translation. Antisense approaches involve the design of oligonucleotides which are complementary to an mRNA
- 20 sequence. The antisense oligonucleotides will bind to the complementary mRNA sequence transcripts and prevent translation. Absolute complementarity, although preferred, is not required.

- A sequence "complementary" to a portion of an RNA, as referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA,
- 25 forming a stable duplex; in the case of double-stranded antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA it may contain and still form a stable duplex (or triplex, as the
- 30 case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

- In one embodiment, oligonucleotides complementary to non-coding regions of the sequence of interest could be used in an antisense approach to inhibit translation of endogenous mRNA. Antisense nucleic acids should be at least six nucleotides in length,
- 35 and are preferably oligonucleotides ranging from 6 to about 50 nucleotides in length. In

specific aspects, the oligonucleotide is at least 10 nucleotides, at least 17 nucleotides, at least 25 nucleotides or at least 50 nucleotides.

Regardless of the choice of target sequence, it is preferred that *in vitro* studies are first performed to quantitate the ability of the antisense oligonucleotide to inhibit sequence expression. It is preferred that these studies utilize controls that distinguish between antisense gene inhibition and nonspecific biological effects of oligonucleotides. Additionally, it is envisioned that results obtained using the antisense oligonucleotide are compared with those obtained using a control oligonucleotide. It is preferred that the control oligonucleotide is of approximately the same length as the test oligonucleotide and that the nucleic acid of the oligonucleotide differs from the antisense sequence no more than is necessary to prevent specific hybridization to the target sequence.

The oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, hybridization, etc. The oligonucleotide may include other appended groups such as peptides (*e.g.*, for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane (see, *e.g.*, Letsinger, *et al.*, 1989, *Proc. Natl. Acad. Sci. U.S.A.* 86:6553-6556; Lemaitre, *et al.*, 1987, *Proc. Natl. Acad. Sci. U.S.A.* 84:648-652; PCT Publication No. WO88/09810, published December 15, 1988) or hybridization-triggered cleavage agents (see, *e.g.*, Krol *et al.*, 1988, *BioTechniques* 6:958-976) or intercalating agents (see, *e.g.*, Zon, 1988, *Pharm. Res.* 5:539-549). To this end, the oligonucleotide may be conjugated to another molecule, *e.g.*, a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

The antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxycarboxymethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (*v*), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (*v*), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

5 The antisense oligonucleotide may also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

5 In yet another embodiment, the antisense oligonucleotide comprises at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

10 In yet another embodiment, the antisense oligonucleotide is an α -anomeric oligonucleotide. An α -anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β -units, the strands run parallel to each other (Gautier, *et al.*, 1987, *Nucl. Acids Res.* 15:6625-6641). The oligonucleotide is a 2'-O-methylribonucleotide (Inoue, *et al.*, 1987, *Nucl. Acids Res.* 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue, *et al.*, 1987, *FEBS Lett.* 215:327-330).

15 Oligonucleotides of the invention may be synthesized by standard methods known in the art, *e.g.*, by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein, *et al.* (1988, *Nucl. Acids Res.* 16:3209), methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin, *et al.*, 1988, *Proc. Natl. Acad. Sci. U.S.A.* 85:7448-7451), *etc.*

20 While antisense nucleotides complementary to the coding region sequence could be used, those complementary to the transcribed, untranslated region are most preferred.

25 Antisense molecules should be delivered to cells that express the SHR sequence *in vivo*. A number of methods have been developed for delivering antisense DNA or RNA to cells; *e.g.*, antisense molecules can be injected directly into the tissue site, or modified antisense molecules, designed to target the desired cells (*e.g.*, antisense linked to peptides or antibodies which specifically bind receptors or antigens expressed on the target cell surface) can be administered systemically.

30 A preferred approach to achieve intracellular concentrations of the antisense sufficient to suppress translation of endogenous mRNAs utilizes a recombinant DNA construct in which the antisense oligonucleotide is placed under the control of a strong pol III or pol II promoter. The use of such a construct to transfect target cells in the patient will result in the transcription of sufficient amounts of single stranded RNAs which will form complementary base pairs with the endogenous sequence transcripts and thereby prevent translation of the mRNA. For example, a vector can be introduced *e.g.*, such that it is taken

Ribozyme molecules designed to catalytically cleave target gene mRNA transcripts can also be used to prevent translation of target gene mRNA and, therefore, expression of target gene product. (See, *e.g.*, PCT International Publication WO90/11364, published October 4, 1990; Sarver, *et al.*, 1990, *Science* 247, 1222-1225).

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. (For a review, see Rossi, 1994, *Current Biology* 4:469-471). The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by an endonucleolytic cleavage event. 25 The composition of ribozyme molecules must include one or more sequences complementary to the target gene mRNA, and must include the well known catalytic sequence responsible for mRNA cleavage. For this sequence, see, e.g., U.S. Patent No. 5,093,246, which is incorporated herein by reference in its entirety.

While ribozymes that cleave mRNA at site specific recognition sequences
30 can be used to destroy target gene mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions which form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in
35 Myers, 1995, *Molecular Biology and Biotechnology: A Comprehensive Desk Reference*, VCH Publishers, New York, (see especially Figure 4, page 833) and in Haseloff and

Gerlach, 1988, *Nature*, 334:585-591, which is incorporated herein by reference in its entirety.

- 5 Preferably the ribozyme is engineered so that the cleavage recognition site is located near the 5' end of the target gene mRNA, *i.e.*, to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

- The ribozymes of the present invention also include RNA endoribonucleases (hereinafter "Cech-type ribozymes") such as the one which occurs naturally in *Tetrahymena thermophila* (known as the IVS, or L-19 IVS RNA) and which has been extensively described by Thomas Cech and collaborators (Zaug, *et al.*, 1984, *Science*, 224:574-578; 10 Zaug and Cech, 1986, *Science*, 231:470-475; Zaug, *et al.*, 1986, *Nature*, 324:429-433; published International patent application No. WO 88/04300 by University Patents Inc.; Been and Cech, 1986, *Cell*, 47:207-216). The Cech-type ribozymes have an eight base pair active site that hybridizes to a target RNA sequence whereafter cleavage of the target RNA takes place. The invention encompasses those Cech-type ribozymes that target eight base- 15 pair active site sequences that are present in the target gene.

- As in the antisense approach, the ribozymes can be composed of modified oligonucleotides (*e.g.*, for improved stability, targeting, *etc.*) and should be delivered to cells that express the target gene *in vivo*. A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive pol III or pol II 20 promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous target gene messages and inhibit translation. Because ribozymes, unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficiency.

- Endogenous target gene expression can also be reduced by inactivating or 25 "knocking out" the target gene or its promoter using targeted homologous recombination (*e.g.*, see Smithies, *et al.*, 1985, *Nature* 317:230-234; Thomas and Capecchi, 1987, *Cell* 51:503-512; Thompson, *et al.*, 1989, *Cell* 5:313-321; each of which is incorporated by reference herein in its entirety). For example, a mutant, non-functional target gene (or a completely unrelated DNA sequence) flanked by DNA homologous to the endogenous 30 target gene (either the coding regions or regulatory regions of the target gene) can be used, with or without a selectable marker and/or a negative selectable marker, to transfect cells which express the target gene *in vivo*. Insertion of the DNA construct, via targeted homologous recombination, results in inactivation of the target gene. Such approaches are particularly suited in the agricultural field where modifications to ES (embryonic stem) cells 35 can be used to generate animal offspring with an inactive target gene (*e.g.*, see Thomas and Capecchi, 1987 and Thompson, 1989, *supra*). However this approach can be adapted for

use in plants provided the recombinant DNA constructs are directly administered or targeted to the required site *in vivo* using appropriate viral vectors.

- Alternatively, endogenous target gene expression can be reduced by targeting deoxyribonucleotide sequences complementary to the regulatory region of the target gene (i.e., the target gene promoter and/or enhancers) to form triple helical structures which prevent transcription of the target gene in target cells in the plant. (See generally, Helene, 1991, *Anticancer Drug Des.*, 6(6):569-584; Helene, *et al.*, 1992, *Ann. N.Y. Acad. Sci.*, 660:27-36; and Maher, 1992, *Bioassays* 14(12):807-815).

- Nucleic acid molecules to be used in triple helix formation for the inhibition of transcription should be single stranded and composed of deoxynucleotides. The base composition of these oligonucleotides must be designed to promote triple helix formation via Hoogsteen base pairing rules, which generally require sizable stretches of either purines or pyrimidines to be present on one strand of a duplex. Nucleic acids may be pyrimidine-based, which will result in TAT and CGC⁺ triplets across the three associated strands of the resulting triple helix. The pyrimidine-rich molecules provide base complementarity to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules may be chosen which are purine-rich, for example, contain a stretch of G residues. These molecules will form a triple helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues are located on a single strand of the targeted duplex, resulting in GGC triplets across the three strands in the triplex.

- Alternatively, the potential sequences that can be targeted for triple helix formation may be increased by creating a so-called "switchback" nucleic acid molecule. Switchback molecules are synthesized in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizable stretch of either purines or pyrimidines to be present on one strand of a duplex.

- In an alternate embodiment, double stranded RNA interference (RNA-i) can be used to specifically disrupt gene expression. More specifically, RNA-i can be used for the targeted disruption of given genetic functions in *Drosophila* (Misquitta, L., *et al.*, 1999, *PNAS* 96:1451-1456), in plants such as *Arabidopsis thaliana* (Chuang, C.F., *et al.*, 2000, *PNAS* 97:4985-4990), etc.

- Anti-sense RNA and DNA, ribozyme, RNA-i and triple helix molecules of the invention may be prepared by any method known in the art for the synthesis of DNA and RNA molecules, as discussed above. These include techniques for chemically synthesizing oligodeoxyribonucleotides and oligoribonucleotides well known in the art such as for example solid-phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the

antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

5.6.3. Transgenic Plants That Express a Transgene Controlled by the *SHR* Promoter

In accordance with the present invention, a desired plant may be engineered to express a gene of interest under the control of the *SHR* promoter. *SHR* promoters and functional portions thereof refer to regions of the nucleic acid sequence which are capable of promoting tissue-specific transcription of an operably linked gene of interest in the embryo, stem, root nodule and/or root of a plant. The *SHR* promoter described herein refers to the regulatory elements of *SHR* genes as described above.

Genes that may be beneficially expressed in the roots and/or root nodules of plants include genes involved in nitrogen fixation or cytokines or auxins, or genes which regulate growth, or growth of roots. In addition, genes encoding proteins that confer on plants herbicide, salt or pest resistance may be engineered for root specific expression. The nutritional value of root crops may be enhanced also through *SHR* promoter driven expression of nutritional proteins. Alternatively, therapeutically useful proteins may be expressed specifically in root crops.

Genes that may be beneficially expressed in the stems of plants include those involved in starch lignin or cellulose biosynthesis.

In accordance with the present invention, desired plants which express a heterologous gene of interest under the control of the *SHR* promoter may be engineered by transforming a plant cell with *SHR* promoter driven constructs using those techniques described, *supra*.

5.6.4. Screening of Transformed Plants For Those Having Desired Altered Traits

It will be recognized by those skilled in the art that in order to obtain transgenic plants having the desired engineered traits, screening of transformed plants (*i.e.*, those having an gene construct of the invention) having those traits may be required. For example, where the plants have been engineered for ectopic overexpression of a *SHR* gene, transformed plants are examined for those expressing the *SHR* gene at the desired level and in the desired tissues and developmental stages. Where the plants have been engineered for

suppression of the *SHR* gene product, transformed plants are examined for those expressing the *SHR* gene product (e.g., RNA or protein) at reduced levels in various tissues. The plants exhibiting the desired physiological changes, e.g., ectopic *SHR* overexpression or *SHR* suppression, may then be subsequently screened for those plants that have the desired structural changes at the plant level (e.g., transgenic plants with overexpression or suppression of *SHR* gene having the desired altered root structure). The same principle applies to obtaining transgenic plants having tissue-specific expression of a heterologous gene in embryos and/or roots by the use of a *SHR* promoter driven expression construct.

Alternatively, the transformed plants may be directly screened for those exhibiting the desired structural and functional changes. In one embodiment, such screening may be for the size, length or pattern of the root of the transformed plants. In another embodiment, the screening of the transformed plants may be for altered gravitropism or decreased susceptibility to lodging. In other embodiments, the screening of the transformed plants may be for improved agronomic characteristics (e.g., faster growth, greater vegetative or reproductive yields or improved protein contents, etc.), as compared to unengineered progenitor plants, when cultivated under various growth conditions (e.g., soils or media containing different amounts of nutrients and water content).

According to the present invention, plants engineered with *SHR* overexpression may exhibit improved vigorous growth characteristics when cultivated under conditions where large and thicker roots are advantageous. Plants engineered for *SHR* suppression may exhibit improved vigorous growth characteristics when cultivated under conditions where thinner roots are advantageous.

Engineered plants and plant lines possessing such improved agronomic characteristics may be identified by examining any of following parameters: 1) the rate of growth, measured in terms of rate of increase in fresh or dry weight; 2) vegetative yield of the mature plant, in terms of fresh or dry weight; 3) the seed or fruit yield; 4) the seed or fruit weight; 5) the total nitrogen content of the plant; 6) the total nitrogen content of the fruit or seed; 7) the free amino acid content of the plant; 8) the free amino acid content of the fruit or seed; 9) the total protein content of the plant; and 10) the total protein content of the fruit or seed. The procedures and methods for examining these parameters are well known to those skilled in the art.

According to the present invention, a desired plant is one that exhibits improvement over the control plant (i.e., progenitor plant) in one or more of the aforementioned parameters. In an embodiment, a desired plant is one that shows at least 5% increase over the control plant in at least one parameter. In a preferred embodiment, a desired plant is one that shows at least 20% increase over the control plant in at least one

parameter. Most preferred is a plant that shows at least 50% increase in at least one parameter.

5.7. Screening Assays

5 Genetically engineered cells, cell lines and/or transgenic plants containing a SHR promoter, or fragment thereof, operably linked to a reporter gene, can be used as systems for the screening of agents that modulate SHR promoter activity. Such transgenic plants provide an experimental model *in vivo* (or can be used as a source of primary cells or cell lines for use *in vitro*) which can be used to develop new methods of treating root and/or
10 shoot-related disorders.

The present invention encompasses screening assays designed to identify compounds that modulate activity of the SHR promoter. The present invention encompasses *in vitro* and cell-based assays, as well as *in vivo* assays in transgenic plants. As described hereinbelow, compounds to be tested may include, but are not limited to,
15 oligonucleotides, peptides, proteins, small organic or inorganic compounds, antibodies, *etc.*

Examples of compounds may include, but are not limited to, peptides, such as, for example, soluble peptides, including, but not limited to, Ig-tailed fusion peptides, and members of random peptide libraries; (see, *e.g.*, Lam, *et al.*, 1991, *Nature* 354:82-84; Houghten, *et al.*, 1991, *Nature* 354:84-86), and combinatorial chemistry-derived molecular
20 library made of D- and/or L- configuration amino acids, phosphopeptides (including, but not limited to members of random or partially degenerate, directed phosphopeptide libraries; see, *e.g.*, Songyang, *et al.*, 1993, *Cell* 72:767-778), antibodies (including, but not limited to, polyclonal, monoclonal, human, humanized, anti-idiotypic, chimeric or single
25 chain antibodies, and FAb, F(ab')₂ and FAb expression library fragments, and epitope-binding fragments thereof), and small organic or inorganic molecules.

In one preferred embodiment, genetically engineered cells, cell lines or primary cultures of germ and/or somatic cells containing a plant SHR promoter operatively linked to a heterologous gene are used to develop assay systems to screen for compounds which can inhibit sequence-specific DNA-protein interactions. Such methods comprise
30 contacting a compound to a cell that expresses a gene under the control of a SHR promoter, or a transcriptionally active fragment thereof, measuring the level of the gene expression or gene product activity and comparing this level to the level of gene expression or gene product activity produced by the cell in the absence of the compound, such that if the level obtained in the presence of the compound differs from that obtained in its absence, a
35 compound capable of modulating the expression of the plant SHR promoter has been

identified. Alterations in gene expression levels may be by any number of methods known to those of skill in the art *e.g.*, by assaying for reporter gene activity, assaying cell lysates for mRNA transcripts, *e.g.* by Northern analysis or using other methods known in the art for assaying for gene products expressed by the cell.

- 5 In another embodiment, microdissection and transillumination can be used. In this embodiment, a test agent is delivered to the transgenic plant by any of a variety of methods. The effect of such test compounds on the root and/or shoot cells can be analyzed by the microdissection and transillumination of the cells. If the level of reporter gene expression observed or measured in the presence of the compound differs from that
10 obtained in its absence, a compound capable of modulating the expression of the plant SHR promoter has been identified.

- In various embodiments of the invention, compounds that may be used in screens for modulators include peptides, small molecules, both naturally occurring and/or synthetic (*e.g.*, libraries of small molecules or peptides), cell-bound or soluble molecules,
15 organic, non-protein molecules and recombinant molecules that may have SHR promoter binding capacity and, therefore, may be candidates for pharmaceutical agents.

- Alternatively, the proteins and compounds include endogenous cellular components which interact with SHR promoter sequences *in vivo*. Cell lysates or tissue homogenates may be screened for proteins or other compounds which bind to the SHR
20 promoter, or fragment thereof. Such endogenous components may provide new targets for pharmaceutical and therapeutic interventions.

- In one embodiment, libraries can be screened. Many libraries are known in the art that can be used, *e.g.*, peptide libraries, chemically synthesized libraries, recombinant (*e.g.*, phage display libraries), and *in vitro* translation-based libraries. In one embodiment
25 of the present invention, peptide libraries may be used to screen for agonists or antagonists of SHR promoter-linked reporter expression. Diversity libraries, such as random or combinatorial peptide or non-peptide libraries can be screened for molecules that specifically modulate SHR promoter activity. Random peptide libraries consisting of all possible combinations of amino acids attached to a solid phase support may be used to
30 identify peptides that are able to activate or inhibit SHR promoter activities (Lam, K.S. *et al.*, 1991, Nature 354: 82-84). The screening of peptide libraries may have therapeutic value in the discovery of pharmaceutical agents that stimulate or inhibit the expression of SHR promoter regions.

- Examples of chemically synthesized libraries are described in Fodor *et al.*,
35 1991, Science 251:767-773; Houghten *et al.*, 1991, Nature 354:84-86; Lam *et al.*, 1991,

5 Nature 354:82-84; Medynski, 1994, BioTechnology 12:709-710; Gallop *et al.*, 1994, J. Medicinal Chemistry 37(9):1233-1251; Ohlmeyer *et al.*, 1993, Proc. Natl. Acad. Sci. USA 90:10922-10926; Erb *et al.*, 1994, Proc. Natl. Acad. Sci. USA 91:11422-11426; Houghten *et al.*, 1992, Biotechniques 13:412; Jayawickreme *et al.*, 1994, Proc. Natl. Acad. Sci. USA 91:1614-1618; Salmon *et al.*, 1993, Proc. Natl. Acad. Sci. USA 90:11708-11712; PCT Publication No. WO 93/20242; and Brenner and Lerner, 1992, Proc. Natl. Acad. Sci. USA 89:5381-5383.

10 Examples of phage display libraries are described in Scott and Smith, 1990, Science 249:386-390; Devlin *et al.*, 1990, Science, 249:404-406; Christian, *et al.*, 1992, J. Mol. Biol. 227:711-718; Lenstra, 1992, J. Immunol. Meth. 152:149-157; Kay *et al.*, 1993, Gene 128:59-65; and PCT Publication No. WO 94/18318 dated August 18, 1994.

15 By way of example of non-peptide libraries, a benzodiazepine library (*see e.g.*, Bunin *et al.*, 1994, Proc. Natl. Acad. Sci. USA 91:4708-4712) can be adapted for use. Peptoid libraries (Simon *et al.*, 1992, Proc. Natl. Acad. Sci. USA 89:9367-9371) also can be used. Another example of a library that can be used, in which the amide functionalities in peptides have been permethylated to generate a chemically transformed combinatorial library, is described by Ostresh *et al.* (1994, Proc. Natl. Acad. Sci. USA 91:11138-11142).

20 The assays of the present invention may be first optimized on a small scale (*i.e.*, in test tubes), and then scaled up for high-throughput assays. The screening assays of the present invention may be performed *in vitro*, *i.e.*, in test tubes, using purified components or cell lysates. The screening assays of the present invention may also be carried out in intact cells in culture and in plant models. In accordance with the present invention, test compounds which are shown to modulate the activity of the SHR promoter *in*
25 *vitro*, as described herein, will further be assayed *in vivo* in cultured cells and plant models to determine if the test compound has the similar effects *in vivo*.

6. Example: SHORT-ROOT EXPRESSION, CLONING AND CHARACTERIZATION

30 6.1. Materials and Methods

6.1.1. Arabidopsis Strains and Growth Condition

shr-1 (in WS ecotype), *shr-2* (Col), and *scr-1* (WS) have been previously described (Benfey *et al.*, 1993, Development 119, 57-70; Scheres *et al.*, 1995, Development 121, 53-62; Fukaki *et al.*, 1998, Plant J. 14, 425-430; Di Laurenzio *et al.*, 1996, Cell 86,
35 423-433). Unstable *shr-3* and *shr-4* alleles (Col) were identified in lines containing the

autonomous maize transposon, En (Wisman et al., 1998, Plant Mol. Biol. 37, 989-999). The *SCR::GFP* line has been described previously (Sabatini et al., 1999, Cell 99, 463-472; Wysocka-Diller et al., 2000, Development 127, 593-603). The growth condition of seedlings and plants has been described previously (Benfey et al., 1993, Development 119, 57-70).

6.1.2. Genetic Crosses

To determine allelism, a heterozygote carrying the *shr-2* allele was pollinated by *shr-3*. In the resulting progeny (n = 30) 34 % showed the mutant phenotype indicating that the two mutations are allelic. The cross was confirmed by amplifying En specific sequences from the mutant plants (Wisman et al., 1998, Plant Mol. Biol. 37, 989-999). A line homozygous for *shr-2* and *gl1* was pollinated by *shr-4*. In the resulting F1 progeny (n = 15) 100 % showed the mutant phenotype indicating that the two mutations are allelic. The cross was confirmed by the presence of normal trichomes on the mutant.

To construct the *shr-1*, *shr-2*, and *shr-3* lines containing *SCR::GFP*, each *shr* allele was pollinated by a plant homozygous for the *SCR::GFP* fusion, and the resulting F1 and F2 plants were self-pollinated to generate F3 plants homozygous for both the fusion and the *shr* mutation. To construct the *shr-1* and *scr-3* lines containing *SHR::GFP*, each mutant line was pollinated by a plant heterozygous for a *SHR::GFP* fusion, and the resulting kanamycin-resistant F1 plants were self-pollinated to generate F2 plants which segregated lines homozygous for each mutation and containing the transgene.

6.1.3. Mapping

The following mapping crosses were performed: *shr-1/Ws* x Col, *shr-2/Col* x *Ler* and *shr-2/Col* x *ap2-10/C24*. In each case homozygous wild-type or *ap2-10* plants were pollinated with *shr* pollen (with the exception of *shr-2/Col* x *Ler* in which *Ler* pollen was used to pollinate *shr-2*), and the resulting F1 plants were self-pollinated to generate F2 plants segregating the *shr* mutation. The rough map position of *shr-1* was obtained using SSLP (Bell and Ecker, 1994, Genomics 18, 137-144) and CAPS (Konieczny and Ausubel, 1993, Plant J. 4, 403-410) markers by analyzing DNAs from 30 to 40 F2 *shr-1* mutant plants resulting from the mapping cross. For fine mapping, DNAs isolated from 74 and 56 F2 *shr* mutants resulting from crosses of *shr-1* and *shr-2* with wild type, respectively, were analyzed for linkage to the marker ngal 107 (Li and Chory, 1997, Cell 90, 929-38). For the cross between *shr-2* and *ap2-10*, F2 plants carrying the recombinant chromosome were selected for kanamycin resistance conferred by the *NPT II* gene in the T-DNA linked to the *AP2* locus (Jofuku et al., 1994, Plant Cell 6, 1211- 1225). After identification of the

recombinant chromosomes, the order of *shr* in respect to the marker loci was determined by three-point analyses with flanking markers. Based on the analysis of 148, 112 and 486 chromosomes derived from the mapping crosses, 2, 4 and 1 recombination events were observed between *shr-1* and ngal 107, *shr-2* and ngal 107 and *shr-2* and *ap2*, respectively.

- 5 The analysis of the recombinant chromosomes with flanking markers indicated that the *SHR* locus is on chromosome IV, between markers *ap2* (0.2 cM) and ngal 107 (2.62 cM).

6.1.4. Cosegregation Analysis and Inverse PCR

- The segregating population representing the progeny of a *shr-3* heterozygote
- 10 was genotyped based on phenotype and a DNA sample was extracted from the same individuals for Southern analysis. An end fragment (generated with oligos: En7631 5'-GGCTCACATCATGCTAGTCC-3' and En8183 5'-GTTGACCGACACTCTTAGCC-3') of the En transposon was used as probe. A band present in all mutants was identified in lanes corresponding to EcoRV digested DNA (Fig. 3a). A band with identical segregation
 - 15 pattern in the population was observed with EcoRI digestion. In this case the fragment size was 2.6 kb, indicating that the plant derived part of the sequence was about 200 bp.

- The 2.6 kb EcoRI fragment was isolated from an agarose gel. Inverse PCR was performed (essentially as described by Long et al., 1993, Proc. Natl. Acad. Sci. USA 90, 10370-10374) using En sequences as a basis with primers 5'-
- 20 TCTATACGAATAAGAGCGTCC-3' (fwd) and 5'-TATTCGCGTCACAATAGTTCC-3' (rev). An amplification product of approximately 500 bp was obtained, subcloned into a pCRII vector and sequenced.

6.1.5. Sequence Analysis

- 25 Sequence of the amplified product was analyzed using the BLAST program (Altschul et al., 1990, J. Mol. Biol. 215, 403-410). It was identical to the end sequence of the BAC F18N2. The 2.8 kb ClaI/EcoRI end fragment containing the *SHR* gene was subcloned into pBluescript (Stratagene) and sequenced. Subsequently the sequence was confirmed by the Arabidopsis Genomic Initiative analysis of chromosome IV (Mayer et al.,
- 30 1999, Nature 402, 769-777).

The *SHR* gene was amplified from the mutant lines using primers that had been designed based on the wildtype sequence. The *shr-2* allele contained an insertion sequence identical to a region from the BAC F2 1117TRB (Accession number; B24674).

6.1.6. Molecular Techniques

RNA gel blot analysis was performed essentially as described (Di Laurenzio et al., 1996, Cell 86, 423-433). A 440 bp fragment representing a region of non-conserved coding region was used as a probe. This probe was also used to screen approximately

- 5 150,000 plaques of a cDNA library made from whole seedlings. RT-PCR was performed with M-MLV reverse transcriptase (Promega) according to the manufacturer's protocol on mRNA obtained separately from roots and shoots of 10 day-old seedlings. The two cDNA species and the amplification products from the RT-PCR were subsequently analyzed by sequencing.
- 10 The 35S::SHR construct was made by placing the protein-coding region of *SHR* between the CaMV 35S promoter and the nopaline synthase polyadenylation sequence. The *SHR*-coding region as well as 31 bp from the 3'UTR were first amplified by PCR with the primers 5'-CAGTCGACTAGTCATATGGATACTCTCTTTAGATTA-3' and 5'-TGTGGAATTGTGAGCCG-3' using the 2.8-kb subclone of the *SHR* genomic region as a
- 15 template. The former primer removed an Spe I site at codon 7 of *SHR*, while creating new Spe I and Nde I sites around the first ATG. These mutations did not alter the encoded amino acid sequence. The latter primer was designed to anneal to downstream vector sequence in the template subclone. The PCR amplified DNA fragment was cloned into pCR2.1 (Invitrogen) and sequenced. The *SHR*-coding region was excised as an Spe I
- 20 fragment and inserted into the Xba I site of plasmid W104. The resulting plasmid was transformed into *Agrobacterium tumefaciens* (LBA4404) and used to transform wild-type *Arabidopsis* plants (Col) by the floral dipping method (Clough and Bent, 1998, Plant J. 16, 735-743).

6.1.7. Histochemical Techniques and In Situ Hybridization

- 25 Histochemical analysis using monoclonal antibodies (JIM13 and CCRC-M2 antibodies) was performed essentially as described (Di Laurenzio et al., 1996, Cell 86, 423-433). For the construction of the *SHR* promoter:: β -glucuronidase (GUS) marker gene line, the 2.5 kb region upstream of the *SHR* translational start site was amplified by PCR using
- 30 the primers: 5'-CGGGATCCAGAAGCAGAGCGTGGGGTTTC-3' (fwd) and 5'-CGGGATCCCTTTTAATGAATAAGAAAATG-3' (rev) (GGATCC BamHI site). The 2.5 kb PCR fragment was inserted into the pCR 2.1 vector using the TA cloning kit (Invitrogen) and, after BamHI digestion, it was subcloned into the BamHI site upstream of the GUS coding region in pBI101 (Clontech). This binary vector was used to generate transgenic
- 35 plants as described above. T1 seeds were collected in separate pools and transgenic plants were selected by planting on media containing kanamycin (50 μ g/ml). GUS staining of the

SHR::GUS line was performed as described previously (Malamy and Benfey, 1997, Development 124, 33-44). In situ hybridization analysis was performed essentially as described in Di Laurenzio et al. (1996), Cell 86, 423-433.

6.1.8. GFP Imaging of Gene Expression

The 2.5 kb region upstream of the *SHR* translational start site was inserted directly upstream of the mGFP5-ER coding region in pBIN, and used to generate transgenic plants as described above. For GFP analysis, roots were counter-stained with 10 μ g/ml propidium iodide (Sigma) and placed on slides in a drop of water. For analysis in embryos, embryo sacs were dissected from siliques and mounted in 50% glycerol in water. GFP fluorescence was imaged with a Leica confocal microscope, and the FITC channel (green: GFP) was overlaid onto the TRITC channel (red: autofluorescence and propidium iodide) to permit identification of the GFP expressing cells.

6.2. Results

6.2.1. *SHORT-ROOT* Is Required for Asymmetric Cell Division and Cell Specification in Ground Tissue

Previous analysis of two *shr* alleles (*shr-1* and *shr-2*) had shown that there is only a single cell layer between the epidermis and the pericycle of primary and secondary roots (Figure 1D) as compared to the normal radial organization of two ground tissue layers, cortex and endodermis (Figure 1C) (Benfey et al., 1993, Development 119, 57-70; Scheres et al., 1995, Development 121, 53-62). Two additional alleles (*shr-3* and *shr-4*) were identified in a screen of Arabidopsis lines containing the autonomous maize En transposon (Wisman et al., 1998, Plant Mol. Biol. 37, 989-999). All four alleles have a similar phenotype - seedlings have highly reduced root growth and darker cotyledons as compared to wildtype (Figure 1B) (with the exception of some *shr-3* and *shr-4* roots which contain revertant sectors).

Longitudinal optical sections indicate the presence of a cell at the normal position of the cortex/endodermal initial and no evidence for a periclinal division occurring in its immediate progeny (compare WT and *shr* in Figures 3D and 3G, 5B and 5C). This indicates that a primary defect in *shr* is disruption of this asymmetric cell division.

To gain insight into the role of SHR in regulating cell specification, the identity of the single mutant ground tissue cell layer present in *shr* roots was determined. It had previously shown that two endodermal markers, production of the Casparian strip, and binding of the JIM13 antibody (Knox et al., 1990, Planta 181, 512-521) do not occur in the mutant layer in *shr-1* (Scheres et al. 1995, Development 121, 53-62; Benfey et al., 1993,

Development 119, 57-70) (compare WT and *shr* in Figures 6B and 6D). Here, the status of two markers normally expressed in the cortex were analyzed. The CCRC-M2 antibody binds to a carbohydrate epitope in the cell walls of epidermis and cortex (Freshour et al., 1996, Plant Physiol. 110, 1413-1429; Di Lorenzo et al., 1996, Cell 86, 423-433). In *shr-1*, antibody staining is detected in the epidermis as well as the mutant ground tissue layer (compare WT and *shr* in Figures 1E and 1F). Expression of an AX92::GUS construct which is specifically expressed in the root cortex was further tested (Dietrich et al., 1992, Plant Cell 4, 1371-1382; Malamy and Benfey, 1997, Development 124, 33-44). In *shr-1*, this construct expresses exclusively in the mutant layer (Figures 1G and 1H). These results indicate that the *shr* mutant layer has attributes of cortex and that *SHR* is necessary for the specification of endodermis cell identity.

6.2.2. Transposon Tagging and Molecular Cloning of *SHR*

The *shr-3* and *shr-4* alleles segregated a variable number (5-15 %) of seedlings with a wild-type appearance suggesting excision of an En element from the *SHR* locus. Analysis of the progeny of several putative revertants indicated segregation ratios close to the expected 3:1 (wild type to mutant) consistent with these being heterozygotes. These two alleles were derived from lines that typically harbor more than ten copies of the En transposon (Wisman et al., 1998, Plant Mol. Biol. 37, 989-999). To identify which En element was inserted into the *SHR* locus, a cosegregation analysis was carried out. A candidate band present in all mutants was identified (Figure 2A). Inverse PCR amplification was used to identify plant sequences flanking the candidate En element. Comparison to the databases revealed identity to a BAC-end sequence (see Materials and Methods).

Sequence analysis of the BAC revealed an ORF whose deduced amino acid sequence has significant homology to GRAS family genes (Figure 2B; Pysh et al., 1999, Plant J. 18, 111-119). To verify the identity of the cloned gene, this region was amplified from *shr-1* and *shr-2* and sequenced. In *shr-1*, a deletion of 50 bp towards the 3' end of the ORF results in a frameshift and premature termination of translation of the putative amino acid sequence (Figure 2C). In *shr-2*, the full sequence for which is shown in Figure 10 (SEQ ID NO:3), there is a deletion of 10 bp followed by an insertion of 431 bp (sequence that is identical to a moderately repetitive sequence) towards the 5' end of the ORF (Figure 2C). This alteration in the DNA sequence would lead to premature termination of the putative peptide. The sites of insertion of the En elements in *shr-3* and *shr-4* were also determined (Figure 2C).

Further support for the identity of the cloned gene was obtained by analysis of the segregation of the *shr* locus-specific En element in the cloned gene in a population of plants representing progeny of a revertant. Excision of the En element cosegregated with the wild type phenotype and no other En elements cosegregated with the mutation. The nature of the molecular defects and the identical phenotype of the four alleles indicates that they are likely to represent the null phenotype.

The protein encoded by the *SHR* locus would be 531 amino acids in length. Figure 9 and SEQ ID NO:2. The nucleotide sequence of the *Arabidopsis thaliana SHR* (SEQ ID NO:1) is shown in Figure 8. The sequence is from 1 to 2825 base pairs. Further, the mRNA spans 1199 to 2794. All of the motifs found in SCR and the other members of the GRAS gene family (Pysh et al., 1999, Plant J. 18, 111-119) are present in SHR. Unlike SCR, there do not appear to be any introns in the *SHR* locus.

6.2.3. *SHR* is Expressed in Stele Tissue

RNA blot analysis revealed two transcripts in wild-type seedlings (Figure 3A, lane 1). The basis for the two transcripts was investigated by isolation and sequencing of two species of *SHR* cDNA. The only difference between the two species was in their 3' UTRs in which one species' polyA tail was 109 bp further downstream than the other. Analysis of the 3' genomic UTRs revealed two putative polyadenylation signals positioned 239bp and 118bp downstream of the putative termination codon. RT-PCR on either shoot or root tissue confirmed the presence of these two RNA species. These results indicate that the two bands in the RNA blot probably represent transcripts with different polyadenylation sites although additional factors such as different lengths of the polyA tails are possible.

SHR expression was analyzed in three *shr* alleles. The *shr-1* allele has the same level of *SHR* RNA as wild type (Figure 3A, lane 2). Transcript levels are severely reduced but still detectable in *shr-1* (Figure 3A, lane 3). In *shr-3*, no transcripts were detected (Figure 3A, lane 4) which may be the result of the large insertion by the En transposon.

To determine the spatial pattern of *SHR* expression, RNA in situ hybridization analysis was performed. By in situ hybridization, expression in the root appeared to be primarily in the stele which comprises the central vascular cylinder and the surrounding pericycle (Figures 3B and 3C). To determine if the localization of *SHR* RNA was regulated at the transcriptional or posttranscriptional level, 2.5 kb of 5' upstream sequence was fused to either the Green Fluorescent Protein (GFP) coding region (Haseloff et al. 1997, Proc. Natl. Acad. Sci. USA 94, 2122-2127) or to the GUS coding region. The *SHR* promoter region conferred expression that was restricted to the stele and extends down

to the vascular initial cells (Figures 3D and 3E). No *SHR* expression was ever observed in the ground tissue cell lineage of the root, suggesting that *SHR* controls radial organization of ground tissue in a non-cell-autonomous manner.

5 Endodermis and cortex are first formed by an asymmetric cell division of the embryonic ground tissue (Scheres et al., 1995, Development 121, 53-62). It has previously been shown that the *shr* mutation results in an absence of this asymmetric cell division (Scheres et al., 1995, Development 121, 53-62). The *SHR::GFP* reporter construct was used to characterize *SHR* expression at various stages of embryogenesis.

At the late globular stage, *SHR::GFP* is expressed in procambium cells in the lower tier which is presumptive stele tissue (Figure 4A). At this stage, *SCR* is expressed in the ground tissue and hypophysis (Wysocka-Diller et al., 2000, Development 127, 593-603). At the triangular stage, *SHR::GFP* expression is observed in the procambium (Figure 4B). *SCR* expression, at this stage is in the adjacent ground tissue and presumptive center cells (Wysocka-Diller et al., 2000, Development 127, 593-603). *SHR::GFP* expression in the procambium/stele is consistently observed at later stages of embryogenesis (Figure 4C, heart stage; Figure 4D, torpedo stages, Figures 4E and 4F, mature embryo). In addition, this expression extends into the cotyledon petiole, in the region of the presumptive cotyledon procambium (Figures 4D and 4F). No *SHR* expression was ever observed in the ground tissue cell lineage or presumptive center cells, consistent with the *SHR* controlling radial organization of ground tissue in a non-cell-autonomous manner during embryogenesis.

6.2.4. *SHR* Is a Positive Regulator of *SCR* Expression

Both *SHR* and *SCR* regulate the asymmetric cell division of the endodermis/cortex initial daughter cell and of the embryonic ground tissue. *SHR* regulates both cell division and endodermis specification, whereas *SCR* appears to regulate primarily the cell division process. As an initial test to determine whether *SHR* might be an upstream regulator of *SCR* in a pathway that results in asymmetric cell division, double mutants were generated and found to have a phenotype identical to *shr-1*. The epistasis of *shr* is consistent with the previous findings and argues against a pathway in which *SCR* would be upstream of *SHR*.

To determine the relationship between *SCR* and *SHR* activity, the effect of the *scr* mutation on *SHR* expression was first examined. From RNA blot analysis the level of *SHR* expression appears to be unaffected by the putative null *scr-1* mutation (Figure 3A, lane 5), indicating that *SHR* expression is not dependent on *SCR* activity. To investigate the spatial distribution of *SHR* expression in *scr* mutant roots, the *SHR::GFP* transgene was crossed into *scr-3*. In *scr-3* roots, *SHR::GFP* expression was still confined to the stele

(Figure 3F), indicating that the establishment of the *SHR* expression pattern is not dependent on *SCR* activity. These results are consistent with the observation that the mutant ground tissue layer in *scr* retains endodermal differentiation attributes (Di Laurenzio et al., 1996, Cell 86, 423-433).

- 5 To investigate the possibility that there might be a regulatory relationship at the transcriptional level with *SHR* upstream of *SCR*, an RNA blot analysis of *SCR* expression was performed in the *shr* background (Figure 5A). The consistently reduced expression of *SCR* in the *shr* mutant background indicates that *SHR* is essential for normal levels of *SCR* expression in the root. To investigate the spatial distribution of the remaining
- 10 *SCR* expression in *shr* roots, a *SCR::GFP* transgene (Wysocka-Diller et al., 2000, Development 127, 593-603) was crossed into *shr*. In all *shr* alleles analyzed, the level of GFP expression was dramatically lowered compared to that in wild type (compare Figures 5B and 5C). Faint expression in the single ground tissue layer and sometimes in the epidermal layer was observable near the meristematic region. No expression was detected
- 15 in more differentiated tissue. *SHR* is, thus, essential for maintenance of *SCR* expression in the promeristem and in more differentiated root cells. The residual level of *SCR* expression detected in the *shr* mutant ground tissue is apparently not sufficient for the correct radial patterning of the ground tissue.

- 20 To determine if *SHR* is involved in an autoregulation pathway, the effect of the *shr* mutation on *SHR* expression using *shr-1* plants containing the *SHR::GFP* transgene was examined. In these plants, expression was still restricted to the stele tissue (Figure 3G) indicating that *SHR* activity is not necessary for the transcriptional regulation of *SHR*.

6.2.5. Cell division and Cell Specification are Uncoupled in Somatic Revertant Sectors

- 25 The genetic instability of the *shr-3* allele was used to investigate the relationship between *SCR* expression, ground tissue cell division and cell specification. Infrequently, *shr-3* seedlings develop indeterminate primary or secondary roots. A relatively rare class of putative somatic sector roots are those with mutant cotyledons and an
- 30 indeterminately growing primary root. These plants were used in the present analysis because the embryonic origins of the primary root are well documented (Mayer et al., 1993, Development 117, 149-162).

- SCR::GFP* was crossed into the *shr-3* background and seedlings with indeterminate primary roots and mutant cotyledons were visualized by confocal laser
- 35 scanning microscopy. In all primary revertant roots, a sector of wild-type ground tissue with GFP expression in the inner of the two layers was observed (Figure 6G and 6H). This

indicates that there is a partial restoration of the wild-type radial pattern. There was complete concordance of longer roots, sectors with divided ground tissue and high-level GFP expression in the inner layer.

- To determine the differentiation status of the primary revertant roots scored for *SCR::GFP* expression, transverse sections were analyzed for the presence of the epitope recognized by the JIM 13 monoclonal antibody (Knox et al., 1990, *Planta* 181, 512-521; Di Laurenzio et al., 1996, *Cell* 86, 423-433). In wildtype, the antibody decorates endodermis and some stele cells (Figures 6A and 6B), while in *shr*, antibody binding is restricted to the stele and is excluded from the mutant ground tissue layer (Figures 6C and 6D). In sections from partially revertant roots, the ground tissue cells that had divided always expressed this marker (Figures 6E and 6F). Occasionally the continuous domain of divided cells was slightly broader than half of the root, although in most cases it was very narrow. Ground tissue cells that had not divided (and did not express *SCR::GFP*) but were decorated by the antibody also were consistently detected (cell with star in Figures 6E and 6F). This indicates that there has been a spatial uncoupling of cell division and cell specification resulting from the partial reversion process.

6.2.6. Ectopic SHR Expression Alters Cell Division and Cell Specification

- To determine if SHR is sufficient for cell division and cell specification when ectopically expressed, the *SHR* coding region was placed under the control of the Cauliflower Mosaic virus (CaMV) 35S promoter, which is constitutively active in most plant tissues (Odell et al., 1985, *Nature* 313,810-812). Analysis of five independent transformants revealed supernumerary cell layers (from 3 to 7 layers) surrounding the stele in the seedling root (Figures 7A and 7B). The number of extra layers varied among the different independent lines and among progeny from a single line and the radial pattern was not always symmetric in the root. In all lines the stele appeared similar to wild type. In longitudinal optical sections it appeared that the additional cell layers were the result of extra cell divisions that originated in the root meristem (Figure 7A).
- To determine if ectopic SHR expression affects cell specification, root sections from 35S::SHR seedlings were stained with the JIM13 antibody. Staining was observed in most of the supernumerary cells in addition to the stele and presumptive endodermis (Figure 7C).

- To determine the spatial localization of *SHR* expression and its affect on *SCR* expression, in situ hybridizations were performed on root tissue from 35S::SHR seedlings. As would be expected with expression driven by the 35S promoter, *SHR* RNA appeared to

be in all tissues (except possibly in the external root cap cells) (Figure 7D). In the meristematic zone of wildtype roots, *SCR* is expressed very specifically in the endodermis (Di Laurenzio et al. 1996, Cell 86, 423-433; Pysh et al., 1999, Plant J. 18, 111-119). In the meristematic zone of 355::SHR roots, *SCR* expression appeared to be in all tissues except for the stele and the root cap. The ectopic expression of *SCR* in the 355::SHR plants is consistent with the hypothesis that SHR is upstream of *SCR* in a pathway that leads to transcriptional activation of *SCR*. Taken together these results indicate that ectopic *SHR* expression causes extra cell divisions and alters cell specification in the root meristem.

6.2.7. SHR Promoter Cloning and Characterization

To determine if the SHR promoter is capable of driving tissue specific expression, a SHR promoter-glucocorticoid receptor translational fusion (SHR-GR) and a SHR promoter-green fluorescent protein translational fusion (SHR-GFP) were made. The sequence of the SHR promoter used in the experiments is shown at Figure 11 (SEQ ID NO:4). As depicted in the figure, the sequence is from 1 to 2502 base pairs. A schematic showing the structure of the SHR-GR and the SHR-GFP is shown at Figure 12A. The methods by which the SHR-GR and the SHR-GFP were made are shown schematically in Figures 12B-12D.

Experiments with these constructs demonstrated that dexamethasone applied to short-root mutant plants that have the SHR-GR transgene causes complete rescue of the root phenotype, i.e., restoration of the endodermal layer. The SHR-GFP construct is also able to rescue short-root mutant plants. This indicates that a SHR promoter fragment is able to rescue the root phenotype similar to the native promoter.

6.2.8. SHR Coding Sequence driven by SCR Promoter

To determine if the SCR promoter is capable of driving tissue specific expression of a SHR coding sequence, a construct containing the SCR promoter upstream of the SHR coding sequence was prepared. Briefly, wildtype, Arabidopsis plants (ecotype Columbia) were transformed with the plasmid pBIH-SCRprom-SHR by the floral dip method. A schematic showing the method by which the plasmid was prepared is shown in Figure 13. After obtaining transformants, the T2 progeny were analyzed. Observation of transverse sections revealed nine layers of cell files between the stele and epidermis in both hypocotyl and root of the transformants, in place of the normal three and two layers in the wildtype hypocotyl and root, respectively. These ectopic cell layers were found to have endodermal characteristics, as determined by suberin staining which detects the casparian

strip and by the JIM 13 antibody which detects an arabinogalactan epitope in some of the ectopic cell layers.

Moreover, transgenic plants containing the SCR promoter-SHR transgene demonstrate additional cell divisions only in the ground tissue (the tissue between the external epidermis and the vascular cylinder), which is where the SCR promoter is active.

Figure 14 shows the results of attaching the promoter region from the SCR gene to the SHR coding sequence. In contrast to the ectopic cell divisions found throughout the outer layers of the root when the SHR gene is driven by the constitutive 35S promoter (left), with the SCR promoter, ectopic divisions appear to be restricted to the ground tissue (right) (the 2nd and 3rd layers in from the outside of the root. Figure 15 shows cross-sections of hypocotyl and root comparing wildtype plants and SCR promoter-SHR transgenic plants. Note that there are up to nine layers where, normally, there are only two between the epidermis and the stele. Figure 16 shows staining for the Casparian strip. Note that in wildtype plants, the Casparian strip is found only in the endodermis, while in the SCR promoter-SHR transgenic plants, there is evidence for additional Casparian strip in the supernumerary layers. Because the Casparian strip functions to impede water flow from the external environment to the vascular tissue of the plant allowing for selective uptake of solutes, the ability to direct specifically the formation of the Casparian strip could be beneficial in drought tolerance, etc.

6.3. Discussion

6.3.1. SHR Is a New Member of the GRAS Family of Putative Transcription Factors

The deduced amino acid sequence of *SHORT-ROOT* places it in the GRAS gene family, whose founding member is *SCARECROW* (Di Laurenzio et al., 1996, Cell 86, 423-433; Pysh et al., 1999, Plant J. 18, 111-119). Motifs including homopolymeric repeat regions, leucine heptad repeats, and putative nuclear localization signals found in all members of this family raised the possibility that they act as transcription factors. Nuclear localization of at least one member of the family (*RGA*) has been demonstrated. (Silverstone et al., 1998, Plant Cell 10, 155-169). Moreover, it has been suggested that within the C-terminus of some GRAS family proteins is a region similar to the SH2 domain of STAT proteins (Peng et al., 1999, Nature 400, 256-261).

In addition to *SCR* and *SHR*, a third member of the GRAS family has been shown to be a possible regulator of radial patterning. The expression pattern of *SHR* is restricted to the endodermis in the root (Pysh et al., 1999, Plant J. 18, 111-119). This indicates that there is a subfamily of GRAS genes that participate in radial patterning.

Another subfamily of GRAS genes which is involved in gibberellic acid signaling has been shown to share a conserved domain (Peng et al., 1997, *Genes & Dev.* *11*, 3 194-3205; Silverstone et al., 1998, *Plant Cell* *10*, 155-169). No conserved domain that might be specific to GRAS genes involved in radial patterning was found in a comparison of SCR and SHR to other members of the family.

6.3.2. SHR is Upstream of SCR

Previously, it had been shown that *shr* does not express endodermal markers in the root (Benfey et al., 1993, *Development* *119*, 57-70; Scheres et al., 1995, *Development* *121*, 53-62). The use of cortex markers revealed that the remaining ground tissue layer has differentiated attributes of cortex. From this, it can be concluded that SHR is required for both the asymmetric cell division that generates cortex and endodermis and for endodermal specification. Because SCR has been shown to regulate primarily the same asymmetric cell division, it was hypothesized that SHR might function upstream of SCR in a common pathway. The epistasis of *shr* to *scr* was consistent with this hypothesis as was the down-regulation of SCR RNA expression in a *shr* background.

Because SHR is a putative transcription factor, the simplest scenario was that SHR interacts directly with the SCR promoter. The expectation then was that SHR would be expressed in the precursor cells of the ground tissue. By in situ hybridization and promoter::marker gene expression, SHR expression is not detectable in the ground tissue lineage. Rather it appears to be expressed exclusively in stele cells. Because the root radial pattern is established during embryogenesis, it was possible that SHR was first expressed in the embryonic ground tissue and then became restricted to stele tissue after germination. The present analysis of embryonic expression was not consistent with this hypothesis. On the contrary, SHR expression was found only in the precursors to the stele.

Expression of SHR in the stele suggested that it might play a role in development of this tissue. However, all cell types present in the wild-type stele appear to be present in *shr* indicating that if there is a phenotype it is subtle.

6.3.3. SHR Participates in Radial Signaling

The fact that SHR is required for ground tissue patterning and transcriptional regulation of SCR but is not expressed in ground tissue cells indicates that it may act in a non-cell autonomous manner. A trivial explanation would be that SHR expression in the ground tissue is just below detection limits. Analysis of revertant sectors in primary roots of the unstable *shr-3* allele which harbors the SCR::GFP construct was not consistent with this hypothesis. In these plants, partial restoration of a two-layer ground tissue was found, with

the inner layer expressing GFP and binding the JIM 13 antibody. Moreover, ground tissue cells that had not divided, did not express GFP but did bind JIM 13 were found. Because this combination of marker expression was found in many roots, it is highly unlikely that this is due to multiple reversion events.

- 5 The simplest explanation of the partial reversion events, consistent with the fate map of Arabidopsis embryo (Mayer et al., 1993, Development 117, 149-162; Scheres et al., 1994, Development 120, 2475-2487), is that reversion results in *SHR* expression in a subset of stele cells. The first division in the embryo proper always occurs vertically before the horizontal divisions that separate the root and shoot poles. This would indicate that the excision event in plants with revertant roots and mutant cotyledons must have taken place after the first vertical division which would result in, at most, reversion of half of the root tissue.

- 10 In this hypothesis, there are different effects on neighboring ground tissue cells depending on their proximity to the revertant tissue. This suggests that either *SHR* regulates differentiation and the asymmetric cell division by two signals with distinct radial mobility or that one process has a higher sensitivity to the radially moving signal than the other process.

- 15 Two alternatives for the non-cell-autonomous regulation of *SCR* expression by *SHR* are possible. First, it is possible that the *SHR* gene product is present only in the stele cells where it is expressed. In this scenario, *SHR* would regulate *SCR* expression through an intermediate that moves from the stele to the ground tissue cell lineage. Alternatively, the *SHR* protein itself might be transported via plasmodesmata from the stele to the ground tissue cell lineage. The latter scenario is supported by evidence that the maize knotted 1 transcription factor can move from one cell lineage to another (Lucas et al., 1995, Science 270, 1980-1983).

- 20 The phenotype of *35S::SHR* roots supports the finding that *SHR* is a positive regulator of root radial patterning. The presence of supernumerary cell layers and altered cell specification in the extra cell layers strongly suggests that *SHR* is both necessary and sufficient for cell division and cell specification in the root meristem.

30

6.3.4. Three-Dimensional Cell Communication in the Root Meristem

- The present results concerning the non-cell autonomous mode of interaction of *SHR* with *SCR* provide new insight into how positional information related to patterning is distributed in the plant meristem. Recently, the role of vascular tissue as a source of positional information has been demonstrated in the context of auxin signaling in organizing the distal pattern of the root (Sabatini et al., 1999, Cell 99, 463-472). *SHR*-mediated radial

patterning of the ground tissue provides further evidence for the role of vascular tissue as playing an important role in organizing plant organogenesis.

For the cortex/endodermal initial cell of the root meristem, van den Berg et al (1995, Nature 378, 62-65; 1997, Nature 390, 287-289) have shown that a "top-down" signal is essential for the asymmetric division of its immediate daughter cell, and that another contact dependent signal from the underlying quiescent center is essential for the maintenance of the undifferentiated status of the initial. The present invention now identifies a new radial signaling pathway, which regulates the asymmetric division of the immediate daughter cell of the initial through the *SHR* and *SCR* genes. This indicates the presence of a three-dimensional signaling network, which controls the developmental status of the initial cell and its derivatives.

The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed since these embodiments are intended as illustration of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference.

WHAT IS CLAIMED IS:

1. An isolated nucleic acid molecule comprising a nucleotide sequence that encodes a short-root protein.
- 5 2. The isolated nucleic acid molecule of claim 1, wherein said short-root protein comprises the amino acid sequence of SEQ ID NO:2
3. An isolated nucleic acid molecule comprising a short-root nucleotide
10 sequence.
4. The isolated nucleic acid molecule of claim 3, wherein said short-root nucleotide sequence comprises the nucleotide sequence of *SHR*, *shr-1*, *shr-2*, *shr-3* or *shr-4*.
- 15 5. An isolated nucleic acid molecule which hybridizes under stringent conditions to the nucleic acid molecule according to any one of claims 1 and 3, or the complement thereof.
6. A recombinant vector comprising the nucleic acid molecule of 5.
- 20 7. An expression vector comprising the nucleic acid molecule of claim 5 operatively associated with a regulatory nucleic acid controlling the expression of the nucleic acid molecule in a host cell.
- 25 8. A genetically-engineered cell comprising the nucleic acid molecule of claim 5.
9. A genetically-engineered host cell comprising the nucleic acid molecule of claim 5 operatively associated with a regulatory nucleic acid controlling the expression of
30 the nucleic acid molecule in a host cell.
10. A method for producing a SHORT-ROOT polypeptide comprising expressing a nucleic acid molecule according to claim 5 in a cell.
- 35 11. An isolated SHORT-ROOT polypeptide.

12. The polypeptide of Claim 10 comprising the amino acid sequence of SEQ ID NO:2.

13. An isolated SHORT-ROOT polypeptide encoded by the nucleic acid molecule of claim 5.

14. An antibody that immunospecifically binds the polypeptide of claim 12.

15. An anti-idiotypic antibody that mimics an epitope of a SHORT-ROOT protein.

16. A plant genetically-engineered to overexpress or underexpress a SHORT-ROOT protein or polypeptide, so that cell division is modified, and root and/or stem development is altered.

17. A plant genetically-engineered to overexpress a SHORT-ROOT protein or polypeptide, so that cell division is increased in roots, resulting in thicker root development.

18. A transgenic plant comprising a transgene having the nucleic acid molecule of claim 5.

19. A transgenic plant comprising a transgene having the nucleic acid molecule of claim 5 operatively associated with a regulatory nucleic acid controlling the expression of the nucleic acid molecule in a transgenic plant cell.

20. The transgenic plant of Claim 17, in which the transgene encodes an antisense nucleotide sequence that suppresses expression of endogenous *SHORT-ROOT* gene product, so that cell division is decreased in roots, resulting in thinner root development.

21. A genetically-engineered plant in which the endogenous *SHORT-ROOT* gene is disrupted or inactivated so that cell division is decreased in roots, resulting in thinner root development.

22. A transgenic plant containing a transgene encoding a gene of interest operatively associated with a *SHORT-ROOT* promoter, so that the gene of interest is expressed in a tissue-specific manner in roots or embryos.

5 23. The transgenic plant of Claim 21, in which the gene of interest encodes a gene product that confers herbicide, salt, pathogen, or insect resistance.

24. A transgenic plant containing a transgene encoding a gene of interest operatively associated with a *SHORT-ROOT* promoter, so that the gene of interest is
10 expressed in shoots.

25. The transgenic plant of Claim 23, in which the gene of interest encodes a gene product that increases starch, lignin or cellulose biosynthesis.

15 26. A plant genetically-engineered to overexpress or underexpress the *SHORT-ROOT* protein so that gravitropism of the stem or hypocotyl is altered.

27. The plant of Claim 25, which is less susceptible to lodging than a wild-type plant.

20 28. A method for identifying a compound which modulates expression of a *SHORT-ROOT* comprising:

- (a) contacting a test compound to a cell that expresses a *SHORT-ROOT*;
- (b) measuring a level of the *SHORT-ROOT* expression in the cell; and
- 25 (c) comparing the level of the *SHORT-ROOT* expression in the cell in the presence of the test compound to a level of the *SHORT-ROOT* expression in the cell in the absence of the test compound;

wherein, if the level of the *SHORT-ROOT* expression in the cell in the presence of the test compound differs from the level of expression of the *SHORT-ROOT* in the cell in the
30 absence of the test compound, a compound that modulates expression of the *SHORT-ROOT* is identified.

29. A method for identifying a test compound capable of modulating root or shoot-specific gene expression comprising:

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- (a) measuring the level of expression of a reporter gene under the control of a SHR regulatory region, or a transcriptionally active fragment thereof, in the presence and absence of said test compound, such that if the level obtained in the presence of the test compound differs from that obtained in its absence, then a compound which modulates root or shoot-specific gene expression is identified.

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ABSTRACT OF THE DISCLOSURE

The structure and function of a regulatory gene, *SHORT-ROOT (SHR)*, is described. The *SHR* gene is expressed specifically in root progenitor tissues of embryos, and in roots and stems of seedlings and plants. *SHR* expression controls cell division of certain cell types in roots and affects the organization of root and stem tissues, and affects gravitropism of aerial structures. The invention relates to the *SHR* gene, *SHR*-like genes, *SHR* gene products, (including but not limited to transcriptional products such as mRNAs, antisense, and ribozyme molecules, and translational products such the *SHR* protein, polypeptides, peptides and fusion proteins related thereto), antibodies to *SHR* gene products, *SHR* promoters and regulatory regions and the use of the foregoing to improve agronomically valuable plants.

2000

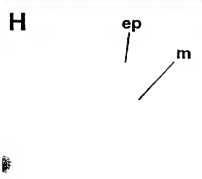
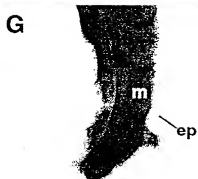
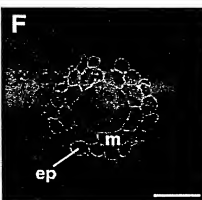
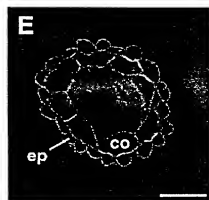
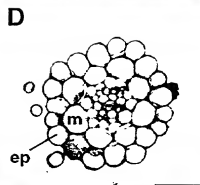
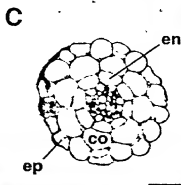
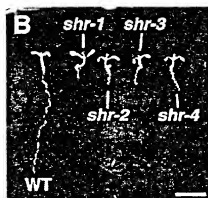
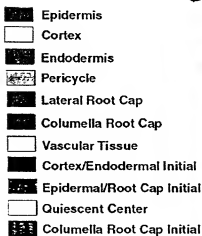


FIG. 2

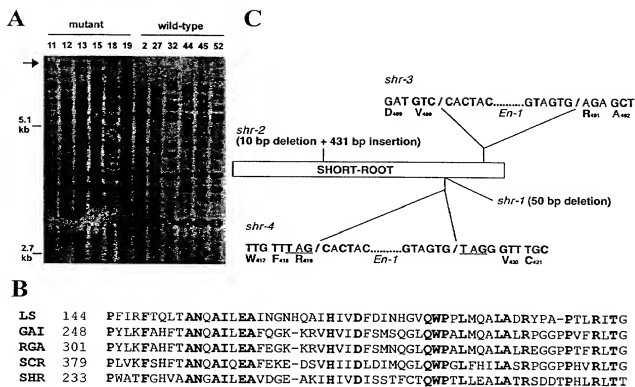


FIG. 3

A

WT *shr-1* *shr-2* *shr-3* *scr-1*

SHR

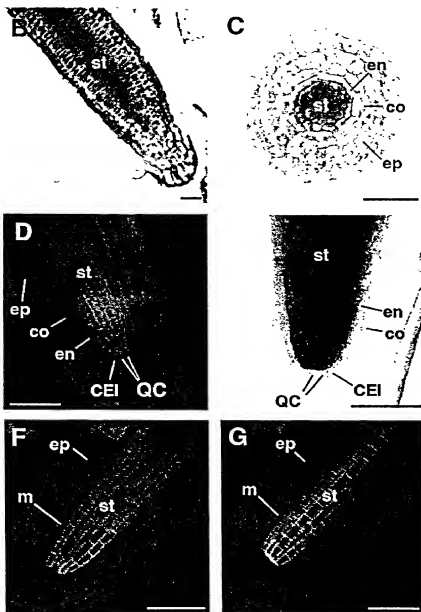


FIG. 4

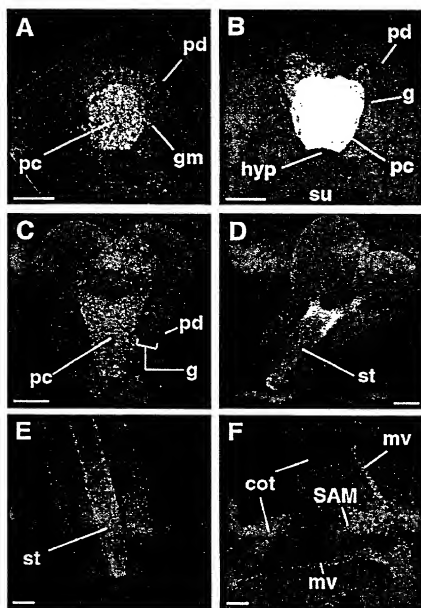


FIG. 5

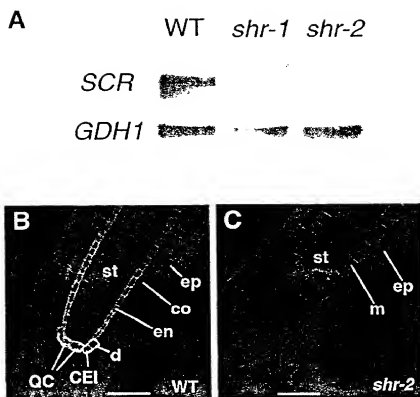


FIG. 6

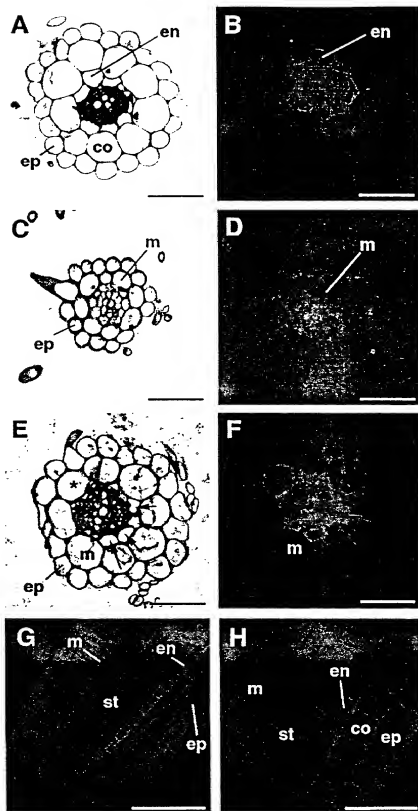
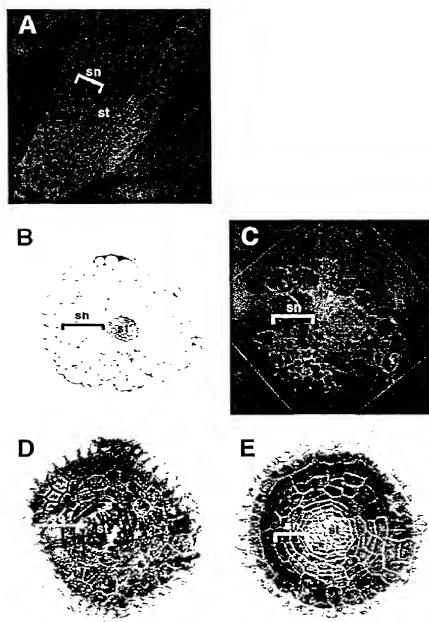


FIG. 7



09573827, 052440
004250, 2882560

FIG. 8

```

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```

FIG. 9

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FIG. 10

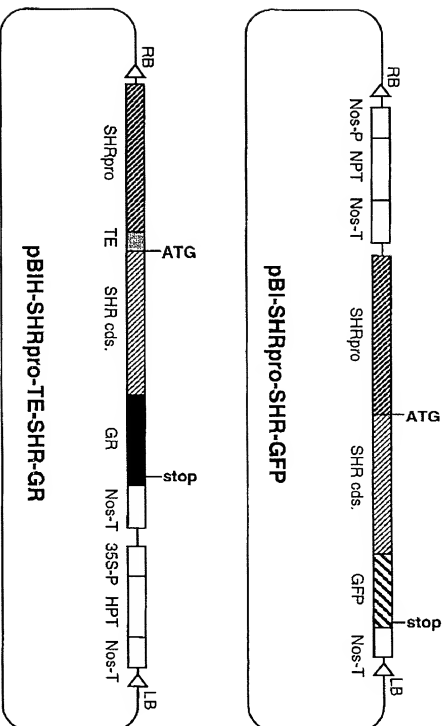
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1981	tggagagatc	agccgggtgt	ttgggctagt	gcgtggcgcc	caacgtaaa	gggtgttttt
2041	attttttcat	aaggaattc				

2.5-kb short-root promoter sequence

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AAACATCTGA	ATTATATACG	GATTTTTTTC	GGAATTTTAC	GCCATATCTG	1600
TAAATATATA	TAACTATGAT	GTCGTTTCCA	AATTCATATG	ATGAACGATC	1650
CACGTAAGTG	CTACTACTCC	TACAAATATG	CATGAGAGAG	ATATGTATTT	1700
ATAAATTTTA	TTTTGAAGAA	GAAATAAGAG	GGAGGTTTAC	TTGGGTGGAT	1750
CGATGTGAAA	ACAAAGAAG	AAAAAGCGAA	ACCCACTAAG	CCATTACATG	1800
ATATCGACCT	TCTTATCTTT	TTCTCTTTTA	TTTTATTTTT	CTCAGGACTT	1850
TTTTCTACTT	AATGAAACCT	CCAAACTATC	TAACATAATC	ACTCCCATGT	1900
AGAATAAAGA	AAATATATA	AGATATTGTT	GATATTTTGT	AACTAGAAAA	1950
TATATTTGCT	CTGTAATTTT	TCGTAAGTTA	AAATCAACAT	TTTCAGTAGA	2000
AACAAATATA	ACTGCAAAAA	GTAGGATCAT	TATTTTGTGC	CAAAATCTCA	2050
GTTAGCTATT	GGGTTGTAGT	AAAAACAAAA	CACATTCTTG	ATTGCCCCCA	2100
AAAAATAAAG	AGAGAGAAGA	ATATTGTTCA	AAAGTGGTCT	CTTCTCTCTC	2150
TAAATATGTT	TTCACTAAAC	CAATTAGATG	TCAACAGTCT	TACAAAGTCC	2200
AAAAAGATAA	CTAGGGACAA	CAATTTCGAT	CAAAAAATCC	TCTTTTTCATG	2250
CTCTTTTTTT	ATTCTCTAGT	CTTTTAAATT	ACTAAATAAA	ACTCAAAAT	2300
CCACCAAAAC	CATTCTCTAC	AACTCACCTT	CATCTAGATT	TACCACTTCC	2350
CACCGAGAAA	CAACAAGAAA	AAAAATATCA	TATATAAAT	TACAAGACAA	2400
CACATGATGC	TGATGCAATA	TACACAACAA	AGTATTAAT	CTTAGATATT	2450
GTGGGTCTCC	CTTTCTTCTA	TTCATTTTCT	TATTCATTAA	AAAAAAAAAA	2500
TG					2502

FIG. 11

FIG. 12A



RB, right border sequence from *Agrobacterium* Ti plasmid
SHRpro, 2.5-Kb 5' upstream region of *SHOOT-ROOT* gene
TE, translational enhancer element of tobacco etch virus
SHR cds., *SHOOT-ROOT* protein coding region
GR, rat glucocorticoid receptor domain coding sequence
GFP, green fluorescent protein coding sequence
Nos-T, transcription terminator of nopaline synthetase gene
35S-P, cauliflower mosaic virus 35S promoter
HPT, hygromycin phosphotransferase coding sequence
NPT, neomycin phosphotransferase coding sequence
LB, left border sequence from *Agrobacterium* Ti plasmid

09578827-0524001

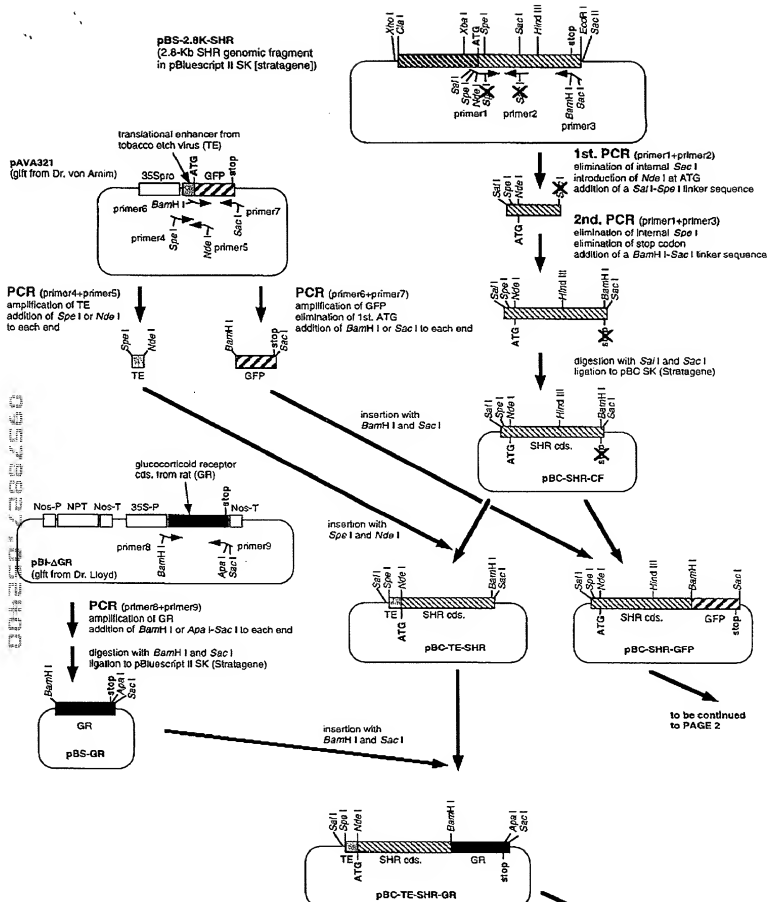


FIG. 12B

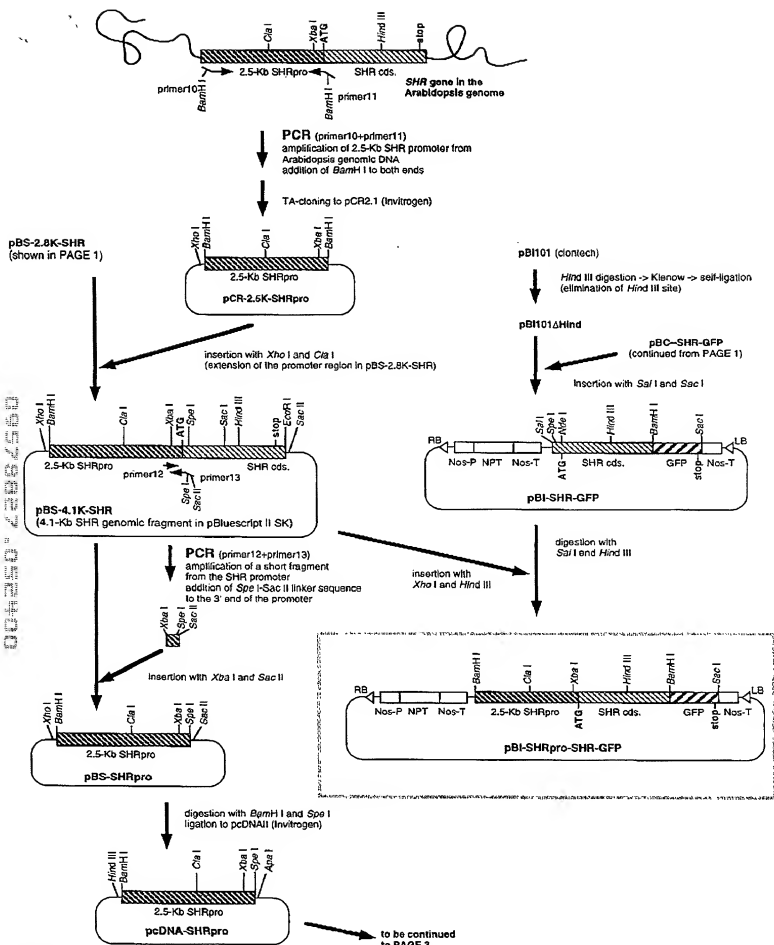
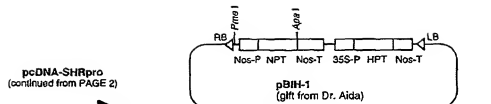


FIG. 12C

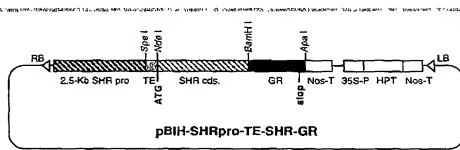
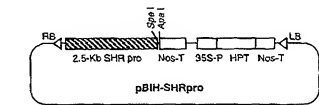


Hind III -> blunting -> Apa I digestion

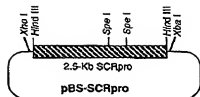
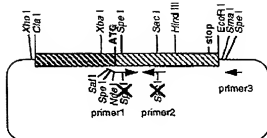
digestion with Pme I (blunt) and Apa I

pBC-TE-SHR-GR
(continued from PAGE 1)

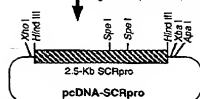
Insertion with Spe I and Apa I



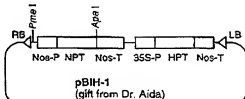
pBS-2.8K-SHR
(2.8-Kb SHR genomic fragment
in pBluescript II SK [stratagene])



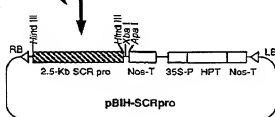
digestion with *Xho I* and *Xba I*
ligation to pcDNAII (Invitrogen)



Xho I → blunting →
Apa I digestion

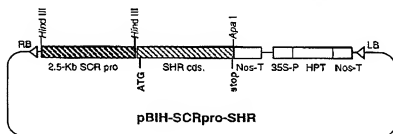


digestion with *Pme I* (blunt) and *Apa I*



digestion with *Xba I* and *Apa I*

Insertion with *Spe I* and *Apa I*



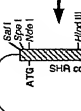
1st. PCR (primer1+primer2)
elimination of internal *Sac I* and *Spe I*
introduction of *Nde I* at ATG
addition of a *Sal I*-*Spe I* linker sequence



2nd. PCR (primer1+primer3)



Cloning to pCR2.1 vector (Invitrogen)



Xho I → blunting → *Sma I* digestion
elimination of a downstream *Spe I* site

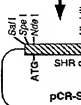
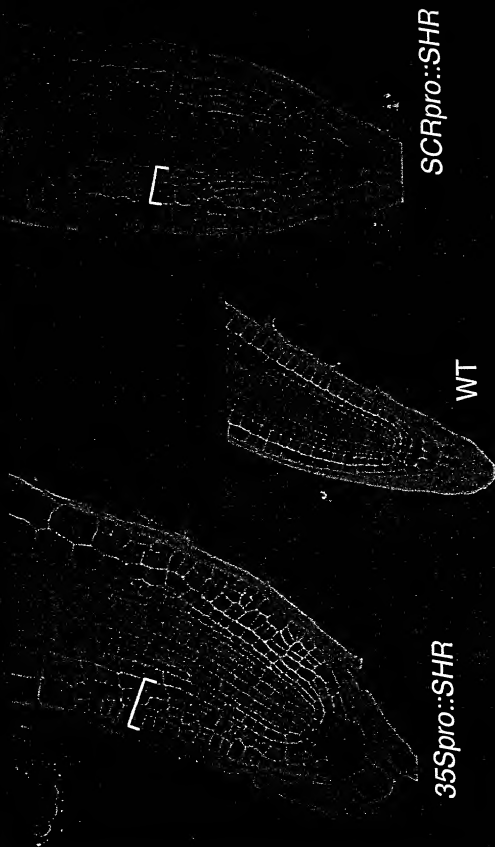


FIG. 13

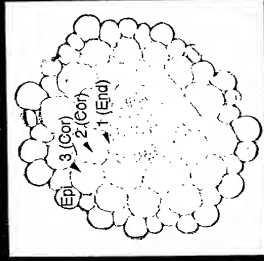


Ectopic *SHR* expression caused abnormal root cell divisions

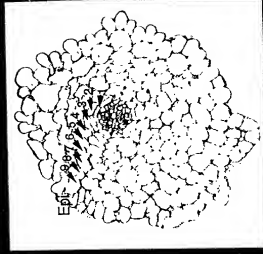
FIG. 14

WT

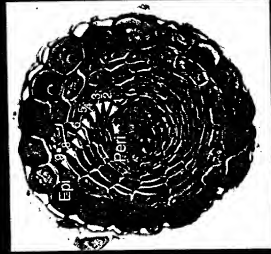
SCRpro::SHR transgenic



hypocotyl



root



Ectopic *SHR* expression under the *SCR* promoter resulted in the indeterminate cell divisions in ground tissue.

FIG. 15

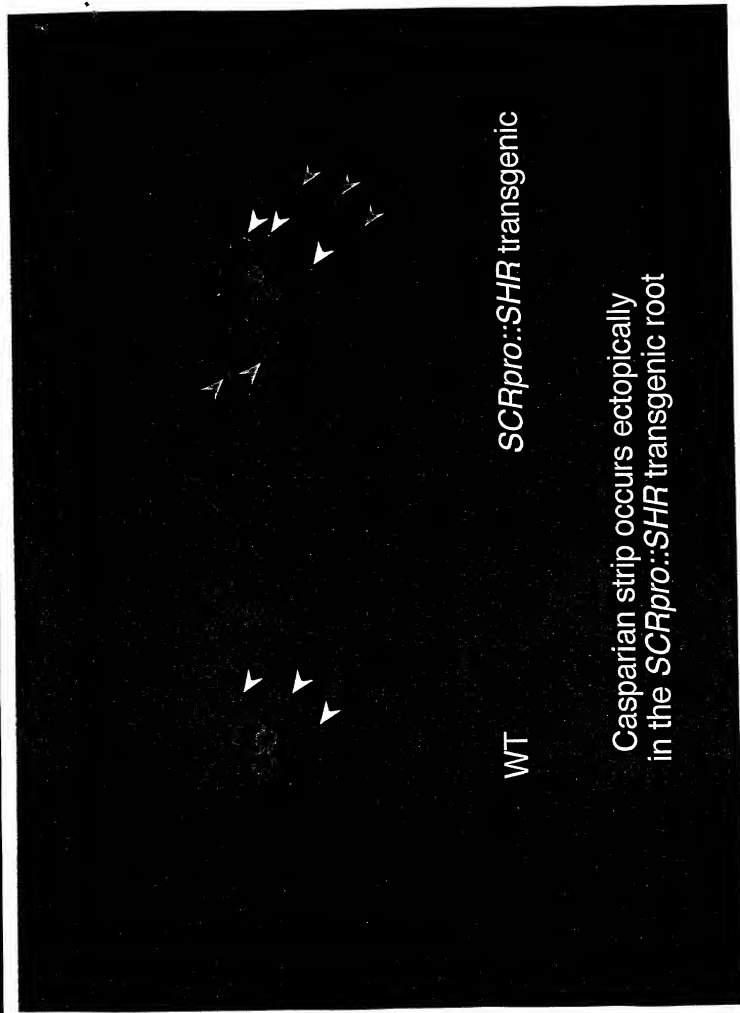


FIG. 16

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below at 201 et seq. underneath my name.

I believe I am the original, first and sole inventor if only one name is listed at 201 below, or an original, first and joint inventor if plural names are listed at 201 et seq. below, of the subject matter which is claimed and for which a patent is sought on the invention entitled

SHORT-ROOT GENE, PROMOTER, AND USES THEREOF

and for which a patent application:

- ☒ is attached hereto and includes amendment(s) filed on (if applicable)
☐ was filed in the United States on as Application No. (for declaration not accompanying application)
with amendment(s) filed on (if applicable)
☐ was filed as PCT international Application No. on and was amended under PCT Article 19 on (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified application, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

EARLIEST FOREIGN APPLICATION(S), IF ANY, FILED PRIOR TO THE FILING DATE OF THE APPLICATION			
APPLICATION NUMBER	COUNTRY	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
			YES <input type="checkbox"/> NO <input type="checkbox"/>
			YES <input type="checkbox"/> NO <input type="checkbox"/>

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

APPLICATION NUMBER	FILING DATE

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION SERIAL NO.	FILING DATE	STATUS		
		PATENTED	PENDING	ABANDONED

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	POST OFFICE ADDRESS	STREET	CITY	STATE OR COUNTRY	ZIP CODE

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201 PHILIP N. BENFEY	SIGNATURE OF INVENTOR 202 YRJO HELARIUTTA	SIGNATURE OF INVENTOR 203 HIDEHIRO FUKAKI
DATE	DATE	DATE
SIGNATURE OF INVENTOR 204 KEIJI NAKAJIMA	SIGNATURE OF INVENTOR 205	SIGNATURE OF INVENTOR 206
DATE	DATE	DATE